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Title: The Use of Comprehensive Molecular Profiling with Network and Control Theory to Better Understand GWI and Model Therapeutic Strategies

PRINCIPAL INVESTIGATOR:
Nancy G. Klimas, M.D.

CONTRACTING ORGANIZATION:
Miami VA Medical Center
Miami, FL 33125

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14. ABSTRACT

The objective of this study is to improve our understanding of GWI pathogenesis in two ways through integration across several of the body's regulatory systems of data and knowledge collected from disparate sources, and by mapping of the coordinated interactions between these physiologic systems and the potential for altered "wiring" of these signaling networks in GWI. Using comprehensive molecular profiling, network and control theory the overarching objective of this proposal is to define the precise nature of these irregularities in immune and neuroendocrine signaling as well as the altered activation states of the corresponding cells such that treatment courses can be designed to redirect the system as a whole to normal pattern of coordinated activity.

Recruitment has been ongoing since July 18, 2011, forty-four patients have been consented to participate in the study; twenty-five of the subjects recruited are symptomatic with GWI and nineteen of the subjects are healthy controls.

Moving beyond a conventional one piece at a time approach, we are using information and dynamic systems theory to identify altered structure and function in endocrine-immune networks. Dr. Broderick, our co-investigator's approach to analyzing the data involves looking not only at parts that might be defective but also how these parts are integrated and regulated in respect to one another. Our current work uses biomarkers in blood to take 'snapshots' of connected networks regulating the body's immune, autonomic and endocrine function. To properly assess these networks, Dr. Broderick has employed statistical tools to quantify the degree of difference between networks in a healthy state versus an illness state. This is leading us to specific pathways that underlie pathogenic immune conversations and we are using our experience in computational biochemistry to conduct detailed investigations of limiting step reactions. Recent findings have suggested that these networks differ in healthy versus ill patients at rest, at peak exercise effort, and post exercise, though the degree and architecture of the differences varies across different stages of the exercise challenge among ill Gulf War veterans. More specifically, networks in ill Gulf War veterans are bigger, not as efficient, and not as centrally organized as those of healthy controls. Of the biomarkers and pathways in this population, 112 were found to be differentially active in Gulf War Illness patients versus healthy controls. The majority of these pathways are immune signaling pathways versus metabolic pathways. Importantly, these models serve to simulate strategies for re-directing immune and endocrine processes using well-chosen sequence of interventions.

15. SUBJECT TERMS

GWI
Comprehensive Molecular Profiling

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Introduction

This annual report sums the work of the past year and briefly reviews the work of the first two years of funding of this project. This study was designed to develop the data base necessary to perfect a computational model of illness and relapse, with the hopes to use this modeling method to propose virtual clinical trials and intervention modeling that could speed up progress towards effective therapy.

We are approaching the completion of the study with 44 of 50 subjects completing an exercise challenge with 9 serial blood draws over 24 hours (25 GWI and 19 GW era healthy controls). These samples have been processed at each time point for immune function, cytokine, cell receptor expression, and neuropeptide expression. They have all had RNA extraction complete, quality assurance studies performed and 180 samples are currently being processed at the Hussman institute using an Affymetrix platform that examines a 60,000 gene platform, using 10 GWI and 10 controls at the 9 time points to assess the optimal dynamic assessment points that best inform the model. The rest of the samples will be processed after these 180 samples have been analysed. Because technologic advances improved the genomic platform, we are also repeating 10 GWI and 10 controls from the pilot sample, though we have 3 points rather than 9 in that sample to analyse. If we can validate that the two platforms provide central and overlapping information, we will tehn be able to use this earlier cohort to strengthen the model further.

Preliminary analyses have been performed on the data thus far and preliminary modles constructed. On the strength of this data set we developed colaborations with two animal modeling groups, that of Mariana Morris (Wright State) and Jim O'Callighan (CDC, toxicology unit) and we completed a network analysis of cytokine expression in a dynamic system such as an exercise challenge and found significant overlaps in the network disruption in the animal models and the human condition. We developed a research consortium, and recently applied for funding to use this mouse modeling system in a pre-clincial trials program, with rapid translation t human studies. Drs. Klimas and Broderick acted as co-PI's in that application.

In July 2012 we also applied for phase I/II funding through the VA Merit Review system to pursue studies of interventions at one key point shown in our model to induce immune and autonomic funding.

We are also analyzing our data for potential biomarkers that could be useful in diagnostics and In intervention studies and have several promising biomarkers identified. As a result we also collaborated in the consortium application submitted by the Boston group (Kim Sullivan PI) with access to large biorepositories to validate these observations.

2012 was a productive year, and over the coming 2 months we will complete the assessments, by the end of October we will have completed the last of the laboratory work, and Dr Broderick's group will be able to concentrate on the final analysis. To that end the VA completed the CRADO agreements allowing the deidentified data set to be transferred to Dr. Broderick's lab for the work requiring access to the super computer and processing hardware available through his computational lab. We have been analyzing our preliminary data, and have several publications, one recent submission we attach to this report. Our investigator group will continue to work on publications and should complete the work of this protocol in the coming year.

To sum the purpose and methods:

Within months after their return from Operation Desert Storm an alarming number of Gulf War veterans began to report a variety of symptoms, including fatigue, musculoskeletal discomfort, skin rashes, and cognitive dysfunction. During deployment, these troops were subjected to a number of potentially hazardous conditions and multiple hypotheses as to the etiology of Gulf War Illness (GWI) have been considered. The symptoms of (GWI) that are most consistently reported include those which are often reported in Chronic Fatigue Syndrome (CFS). The objective of this study is to improve our understanding of GWI pathogenesis in two ways; by integration across several of the body's regulatory systems of data and knowledge collected from disparate sources, and by mapping of the coordinated interactions between these physiologic systems and the potential for altered "wiring" of these signaling networks in GWI. Using comprehensive molecular profiling, network and control theory the overarching objective of this proposal is to define the precise nature of these irregularities in immune and neuroendocrine signaling as well as the altered activation states of the corresponding cells such that treatment courses can be designed to redirect the system as a whole to normal pattern of coordinated activity. To accomplish this goal, we will assess 25 GWI veterans and 25 match health GW era controls using a comprehensive microarray of gene expression, cytokine expression, flow cytometric assessment of subsets, activation state and density of receptor expression, and a key series of neuropeptide concentrations.

Body

The study was approved by the local IRB following the changes that were made, recommended by the DOD, to meet their requirements. Recruitment is ongoing and as of July 18, 2011, forty four subjects have been consented to participate in the study; twenty five of the subjects recruited are symptomatic with GWI, Nineteen of the subjects are healthy controls. The study suffered a number of administrative slowdowns, now resolved. Our 2010 kick off was delayed due to IRB issues with the VA and DoD. There was another delay interupting the exercise challenge component of the study due to new local requirements, documenting and approvals through several committees on the standard operating procedures for the sterilization of our reusable equipment, which consist of a mask attached to the equipment that measures expired gases. This was a nationwide VA initiative that was implemented in early 2010. Exercise challenge testing was halted on Novemeber 28, 2011 through December 9, 2011 due to exercise equipment malfunction and for the completion of maintenance procedures on the equipment used for these test. A final approval was issued on December 9, 2011 for the sterilization of our reusable equipment.Since the approval, recruitment of subjects and study procedures was rapid and we have nearly completed this process, with allof the GWI subjects run and the last of the matched controls to be run in August and early September. We are presently working with Southern Command in effort to recruit the six healthy controls that are missing.

The 44 subjects each have 9 samples , all have been run for cell function, ctytokine, flow cytometry, NPY, and other neuropeptide studies. 180 of these samples are at the Hussman institute and are being processed for gene expression using the Affymetrix platform, then validation using a set of genes on the Nanostring platform. All of the samples have had the RNA extractions and quality control studies needed for genomic studies, in a way that would allow micrRNA or whole genome

studies should funds become available. We have also provided samples to Dr. Richard Sutton for his pathogen discovery project, also funded by the DoD.

Key Research Accomplishments

- Performed novel analysis of pathway activation in Gulf War Illness (GWI) and chronic fatigue syndrome (CFS) subjects during exercise based on gene expression data generated under the preliminary VA-funded pilot project supporting this application. This work has been submitted and is currently under review by the journal Brain, Behavior and Immunity (Elsevier publishing) (see appendix; ref. 1).

- Currently completing a first analysis of the expression profiles of 16 cytokines measured in plasma from Gulf War Illness (GWI) and chronic fatigue syndrome (CFS) subjects during an exercise challenge (A. Smylie; Broderick lab at Univ. of Alberta). Initial results point to significant gender differences between immune response signatures in male and female GWI subjects as well as with CFS subjects.

- Completed first analysis of the alignment between the cytokine signatures from human (male) GWI subjects and cytokine profiles from mouse models of exposure to chemical agents and immune antigens under stress developed at the CDC (Morgantown) and Wright State University. This analysis was also used in support of a CMDRP Consortium grant application invited by the DoD.

- Exploiting synergy with a CMDRP sister project (W81XWH-10-1-0774; Broderick PI), cytokine data from GWI and CFS subjects was used to validate a first model linking regulation of the immune response with the stress response axis and regulation of sex hormones (Craddock et al., 2012; in preparation). This same data was also used to explore alignment of GWI with complex behavior in a detailed model of the immune and fight-or-flight axis (Fritsch et al., 2012; in preparation).

- Completed book chapter (Springer Verlag publishing) describing in vivo (plasma) and in vitro stimulated cytokine response to exercise in GWI subjects using a multivariate time course regression model (ref. 2).

Reportable Outcomes

We have been using a preliminary data set, supported by a VA Merit, to develop the database and informatics work needed for this work. The investigators have been developing the analytic platform based on preliminary work with the following preliminary findings:

- Analysis of pathway activation based on gene expression indicated that GWI was characterized by increases in neuroendocrine-immune signaling and inflammatory activity cast against decreases in apoptotic signaling. Conversely, cell cycle progression and immune signaling were broadly subdued in CFS.

- In 27 symptom and demographic variables measured in GWI subjects, 17 correlated directly with the activity of at least one pathway in one of the 3 exercise phases. Interactions with NF-κB and associated pathways that are consistent with altered cholinergic signaling were the dominant theme underlying symptom expression for GWI.

- Molecular profile describing immune interaction with stress response and sex hormone regulation in male GWI subjects lies in close proximity (60% alignment of immune-endocrine markers) to a naturally available stable state characterized by low testosterone and elevated cortisol at rest. Alignment of this physiological model with GWI biomarker profiles increases to 80% agreement when a detailed immune cell-signaling network is used. These first coarse-grained models suggest that some form of natural protective program is being recruited in these subjects.

- Analysis of fold-change (FC) across 11 cytokines indicated that a mouse model consisting of challenge with LPS potentiated by stress response induced via cortisol doping produced the best fit with cytokine expression in human GWI subjects whether at rest, maximum VO2 or post-exercise ($d2<0.70$). The mMIP-2 mouse surrogate for IL-8 performed better overall than the mKC surrogate in this regard. Inclusion of minocycline in the protocol only improved the degree of concordance, which was most evident at 6 and 12 hours post-exposure.

In addition we have accomplished the following in 2012:

- Continued recruitment of the cohort, currently 44 of 50 completed recruitment, assessment
- Exercise challenge performed on the study subjects, with sample collection at two baseline and 7 times over 24 hours.
- Staff change in January 2012 necessitated informed consent change and new training
- IRB approval for Consent Form changes reflecting staff change on February 6, 2012
- Re Approval of Health Research Ethics Board March 22, 2012 expires April 10, 2013
- Approval to conduct International research on March 27, 2012
- Preliminary data presented to the VA for Research Week on Aril 25, 2012
- Continuing IRB approval May 11, 2012
- Preliminary data presented to a regional GWI conference at the Palo Alto VA, Palo Alto, CA July 2012
- Invited to participate in PW investiators meeting VACO, Washington DC September 2012

Conclusions

We have made extensive progress and find the systems biology is indeed yielding significant new understandings of the complex illness, GWI. We have been using a preliminary data set, supported by a VA Merit, to develop the database and informatics work needed for this work. The investigators have been developing the analytic platform based on preliminary work and have published 3 papers based on these analyses. Preliminary data was presented at the GWI VA RAC in June of 2011, IACFS/GWI meeting in Spetember 2011 (Ottawa, Canada and GWI providers conference on July 2012 (Palo Alto, CA) showing remodeling of regulatory networks and significant differences in metabolic and signaling pathways.

Our preliminary analyses have yielded the data to support three recent submissions, two focused on translatrional research with phase I/II trials, using dynamic modeling before and after interventions that impact targeted mediators to test the intervention's ability to "reset" regulatory networks towards normal dynamics. We will complete recruitment and assessment of the entire cohort over the next 2 months, and complete the genomic studies to complete the laboratory data set before the end of 2012. The computational work is ongoing, but on the completion of the data set, Dr Broderick's computational lab will refine the model and our key publications will be submitted in winter and spring of 2013.

References

1) Broderick G, Ben Hamo R, Vashishtha S, Efroni S, Nathanson L, Barnes Z, Fletcher MA, Klimas NG. Altered Immune Pathway Activity under Exercise Challenge in Gulf War Syndrome. Brain Behav Immun. 2012. Under review.
2) Broderick G, Fletcher MA, Gallgher M, Barnes Z, Vernon SD, Klimas NG. Exploring the Predictive Potential of Immune Response to Exercise in Gulf War Illness. In: In: Yan Q. (Ed.), Psychoneuroimmunology: Methods and Protocols (Methods in Molecular Biology), Springer, New York, NY, In Press.
3) Smylie AL, Broderick G, Fernandes H, Barnes Z, Fletcher MA, Klimas NG. Gender-specific Signatures of Immune Response to Exercise in Gulf War Illness and Chronic Fatigue Syndrome. 2012. In Preparation.
4) Craddock TJA, Miller DB, Fletcher MA, Klimas NG, Broderick G. Towards an Integrative Model of Complex Stress-Mediated Illnesses: Gulf War Illness and Chronic Fatigue Syndrome. 2012, In preparation.
5) Fritsch P, Craddock TJA, Smylie AL, Folcik Nivar VA, Fletcher MA, Klimas NG, de Vries G, Broderick G. A Study of Multiple Homeostatic Regimes in a Discrete Logic Model of Immune Signaling. 2012. In preparation.

Appendices

Appendix A: Journal Article

Klimas NG, Broderick G, Fletcher, MA. Biomarkers for chronic fatigue. Brain Behavior, and Immunity. June 2012, In press.

Appendix B: Journal Submission

Broderick G, Ben Hamo R, Vashishtha S, Efroni S, Nathanson L, Barnes Z, Fletcher MA, Klimas NG. Altered Immune Pathway Activity under Exercise Challenge in Gulf War Syndrome. Brain Behav Immun. 2012. Under review.

Appendix C: Proceedings

1) Altered immune functions in Gulf War illness and potential therapies
   Dr. Nancy Klimas Miami VA Medical Center,
   June 27, 2011 GWI VA RAC Washington DC

2) From Cytokines to Cells to Gene Expression: An Integrative Approach to the Study of Gulf War illness Dr. Gordon Broderick University of Alberta
   June 27, 2011 GWI VA RAC Washington DC

3) Miami VA Research Week, Main Presenter: Nancy Klimas, M.D.
   25th of April, 2012, Miami, FL

4) Gulf War Illness Providers conference. Nancy Klimas, M.D., presenter
   July 2012, Palo Alto VA, Palo Alto, CA

Appendix F: Nancy Klimas, M.D. Curriculum Vitae

CURRICULUM VITAE

1. **Date**: July 2012

2. **PERSONAL STATEMENT** I server PI of this study, a Professor of Medicine at Nova Southeastern University, a diplomate of the American Board of Internal Medicine, a diplomate in Diagnostic Laboratory Immunology, a staff physician and Director of Clinical Immunology Research at the Miami VAMC. I have achieved international recognition for my research and clinical efforts in multi-symptom disorders, chronic fatigue syndrome (CFS), gulf war illness (GWI) and fibromyalgia. I am a past president of the International Association for CFS and Myalgic Encephalopathy (IACFS/ME), a professional organization of clinicians and investigators, and a member of the Health and Human Services (HHS) CFS Advisory Committee. My Veteran's Administration (VA) 8/8ths position protects at least 60% of my effort for university and VA based research. My responsibilities for this grant include selection and referral of all study participants, interpretation of data, generation of manuscripts for publication and dissemination of findings as well as coordination and execution of research efforts in collaboration with other PIs and co-investigators, attending all meetings and monthly conference calls with researchers, support staff and senior investigators. I will utilize established protocols and take necessary security measures including the informed consent process, in which I will hold the key paper file that identifies all participants to a study number that will be kept in a locked file in a locked office.

2a. **Name**: Nancy Grace Klimas

2b. **Home Phone**:

2c. **Home Address**:

2d. **Citizenship**: US

2e. **Visa Type**: None

2f. **Non-Academic Employment**: None

2g. **Military Service**: None

3. **ACADEMIC EMPLOYMENT:** Professor of Medicine at Nova Southeastern University

3a. **Office Address**: VA Medical Center (111-I)
Department of Medicine
University of Miami School of Medicine
1201 NW 16th St
Miami, Florida 33125

3b. **Office Phone**: (305) 575-3267

3c. **Current Academic Rank**: Professor, tenured

3d. **Primary Department**: Medicine

3e. **Academic Appointments**:

2011- present: Professor, Nova Southeastern University College of Osteopathic Medicine, Davie, FL

2011- present Chair, Department of Clinical Immunology, COM, Nova Southeastern University, Davie, Fl

2011- present: Director, NSU Institute for Neuro-Immune Medicine, NSU, Davie FL

2012 - present; Medical Director, NSU Neuro-Immune Medicine Clinics

2011 - present: Professor Emeritus, Departments of Medicine, Psychology, Microbiology

2001 - present: Director of CFS and GWI Research, Miami VA Medical Center

1996 - 2011: Professor of Medicine, University of Miami Miller School of Medicine, Miami, FL

1997 - 2011: Professor of Psychology, University of Miami College of Arts and Sciences

1999 - 2011: Professor of Microbiology and Immunology, University of Miami Miller School of Medicine

1987 - present: Director of AIDS Research, and Co-Director of the AIDS Clinical Research Unit, Miami VA Medical Center

1999 - present: Director, CFS/GWI Multidisciplinary Research Center (initially funded by NIH U01 AI45940)

1985 - present: Co-Director, E.M. Papper Clinical Immunology Laboratory, Division of Rheumatology and Immunology, Department of Medicine, University of Miami School of Medicine

1987 - present: Director, University of Miami Diagnostic Allergy and Immunology Clinic

1984 - 2011: Instructor, Assistant, Associate & Professor. Dept of Medicine, University of Miami

1985-1993, 2000 - present; Director, VA Allergy Clinic

2001 - 6 month sabbatical, Centers for Disease Control and Prevention, National Center for Infectious Diseases/ Division of Viral and Rickettsial Diseases, Viral Exanthems and Herpesviruses Branch, "Emperic Case Definition of CFS" working group.

1998 - 2000: Director, West Palm Beach VAMC Allergy Clinic

1991 - 1996: Associate Professor of Medicine, University of Miami School of Medicine

1994 - 1999: Associate Professor of Microbiology & Immunology, University of Miami School of Medicine

1993 - 1997: Associate Professor of Psychology, University of Miami College of Arts and Sciences

1985 - 1991: Instructor and Assistant Professor of Medicine, University of Miami School of Medicine

1986 - Coordinator, Miami VA Medical Center AIDS Program

1984 - National Cancer Institute Research Fellow; Clinical Immunology Lab, Department of Medicine, University of Miami School of Medicine

4. **HIGHER EDUCATION**

4a. **Institutional**:

Virginia Commonwealth University 9/1972 - 6/1973

University of South Florida, 9/1973 - 6/1976

University of Miami, M.D., 8/1976 - 6/1980

Baylor University Hospitals and Clinics, Medical Internship, 6/1980 – 6/1981

University of Miami Hospitals and Clinics, Medical Residency, 6/1981 – 6/1983

NCI Fellow; Post-Doctoral Fellowship in Diagnostic Laboratory Immunology, E.M. Papper Laboratory of Clinical Immunology, University of Miami, 6/1983 - 6/1984.

5. **PROFESSIONAL ACTIVITIES**

5a. **Certification, Licensure**:

National Boards Part I, II and III

Florida State Medical License, 1983- current (ME41879)

American Board of Internal Medicine, 1984

ABIM Certification in Diagnostic Laboratory Immunology, 1986

Current hospital affiliations:

Miami VAMC, University of Miami Hospitals & Clinics, Jackson Memorial Hospital

5b. **Editorial Responsibilities:**

Founding Editor: Journal of Chronic Fatigue Syndrome
Haworth Press (1992-2001)
Editorial Board, Journal of Chronic Fatigue Syndrome (2001 – current)
Ad hoc reviewer NEJM
Ad hoc Reviewer, Journal of Clinical Immunology
Ad hoc Reviewer, JAMA
Ad hoc Reviewer, Annals of Internal Medicine
Ad hoc Reviewer, AIDS
Ad hoc Reviewer, JAIDS
Ad hoc Reviewer, Psychosomatic Medicine
Ad hoc reviewer, Brain Behavior and Immunity

5c. **Professional and Honorary Organizations**:

International Association for CFS (previously AACFS), Current President
American Society for the Advancement of Science;
Association of Medical Laboratory Immunologists
Clinical Immunology Society
Association of Women in Science;
American Medical Woman's Association
University of Miami Medical Women
Miami Medical Women's Association (VP)

5d. **Honors**:

2004 – Honorary Degree, University of Catalonia, Barcelona Spain.
1998 - Fellow - Academy for Behavioral Medicine Research

1992 - Iron Arrow (University of Miami Honor Association)
1982 - Finalist Beecham Award, Southern Blood Club
1982 - Finalist Burroughs-Wellcome Young Investigator Award
1983 - Southern Medical Association Research Award
1984 - National Research Service Award
1985 - American Cancer Society Institutional Research Award

5e. **Other Professional Activities**:

Miami VAMC AIDS Clinical and Research Unit Developed and wrote the proposal for the Miami VAMC AIDS Clinical and Research Unit, which was one of 3 selected for funding. The proposal included 3 million dollars in construction funds as well as infrastructure support. This resulted in one of the top VA HIV/AIDS clinical and research programs in the US, which is still in operation. Dr Klimas is Director of AIDS Research and Co-director of the Clinical HIV/AIDS program at the Miami VAMC. 1987 to present

University of Miami and VAMC CFS and GWI Research Center : Initially funded with an NIH center grant, ans since supported with NIH , VA, DOD, and private foundation grants, the center is a clinical , translational, and basic science center that integrates research across disciplines. Current studies include genomics, immune, neuroendocrine studies , a anural history study, and clinical trials. Dr. Klimas is Center PI and coordinates the research efforts of four research groups.

Canadian Government advisor in the development of the Clinical Case Definition for Chronic Fatigue Syndrome/Myalgic Encephalitis 2001, which is being revised in 2008.

Whittemore Peterson Institute - This University of Nevada institute is located in Reno, and is in its start up years, developing a clinical research program and a comprehensive clinic for patients with CFS/ME. Dr klimas has been advising its executive committee on long term research goals. The institute is constructing a 25 million dollar facility which should open its doors in 2010.

6 month sabbatical CDC 2001 –, Molecular Epidemiology Program Viral Exanthems and Herpesvirus Branch, Developed the international protocol currently underway to define empirically CFS.

IACFS/ME
Dr. Klimas served as President of the International Association for Chronic Fatigue Syndrome (A national professional organization of investigators and clinicians) from 2005-2007 and was re-elected for another 3-year term in January of 2007. She organized the IACFS conference in Fort Lauderdale in January, 2007, and the conference in Reno Nevaa in March 2009. Each of these conferences were attended by 400 patients and 350 professionals, and provided a unique opportunity for patients to meet and talk with leading international researchers and clinicians.

CFSAC: Chronic Fatigue Syndrome Advisory Committee
In 1996, Secretary for Health Donna Shalala chartered a special committee to advise the Department of Health and Human Services (DHHS) on policy regarding chronic fatigue syndrome (CFS), also known as chronic fatigue and immune dysfunction syndrome or CFIDS or Myalgic Encepahalomyelopathy (ME). This committee, known as the DHHS Chronic Fatigue Syndrome Coordinating Committee (CFSCC), brought together officials representing various health agencies together with seven appointed members of the public to improve coordination of federal CFS programs. A year 2000 review of federal activities on CFS conducted by the General Accounting Office prompted several changes. Among them was the replacement of the CFSCC with a new committee, the CFS Advisory Committee (CFSAC), whose structure more closely matched other DHHS advisory bodies. Secretary Michael Leavitt most recently renewed the charter on August 30, 2006.

Nancy.Klimas served on this committee from 1997 to 2000. She was reappointed to another three-year term on the committee in 2007, which has been extended to 2011.

National Press Club in Washington, DC: The CDC's Chronic Fatigue Syndrome Public Education and Awareness Campaign.

Nancy Klimas was a participant at this event held on November 3, 2006. Present were Julie Gerberding, CDC, John Agwunobi, HHS, William Reeves, CDC and Anthony Komoroff, Harvard. Dozens of reporters from national and local media outlets across the United States were in attendance, and many others participated via phone link. Dr. Klimas remarked, "Historically, the lack of credibility afforded this illness has been a key obstacle to understanding it. Today, with solid evidence that CFS has identifiable biologic underpinnings, and with evidence that people with CFS experience a level of disability equal to that of patients with multiple sclerosis, advanced HIV disease and undergoing chemotherapy, I hope we can begin to put an end to the stigma surrounding this illness." Dr. Klimas also focused on treatments, saying, "Although there's no single treatment—no hoped for 'magic bullet'—that fixes the illness at its core, there are treatments that can improve symptoms, increase function and allow CFS patients to engage in activities of daily living. Current best practices for clinical care include a combination of symptom management, activity management and exercise therapies."

Chronic Fatigue Centers for Research and Clinical Care: A newly conceived program hoping to use clinical care templates to help diagnose and manage complex CFS/ME cases while collecting research data and developing the patient base for clinical trials work. Dr. Klimas is the senior clinician developing the templates the first clinic to implement this format is the Chronic Fatigue Center in Kendall, FL which opened in 2010.

5f. **Consultantships**

1987 - 1990 - VA National AIDS Steering Committee

1987 - 1990 - VA National AIDS Research Subcommittee

1987 - 1991 - VA Train the Trainer National AIDS Education Program

1988 - Present - VA National AIDS Prevention and Counseling Training Program

1988 - Present - Special Review Committee, National VA AIDS Prevention and Education

1990 – 2000 - VA National HIV Therapeutics Advisory Committee

1991 - 2000 - Board of Directors, American Association for Chronic Fatigue Syndrome (An international professional organization of investigators and clinicians).

2002 – present - Board of Directors, American Association for Chronic Fatigue Syndrome (An international professional organization of investigators and clinicians).

1992 - Chairperson of the Program Committee for the First International Meeting: Chronic Fatigue Syndrome, held in Albany NY, sponsored by the AACFS, NIH and CDC.

1994 - Local Coordinator and Program Committee member for the Second International Meeting: Chronic Fatigue Syndrome research Conference, held in Ft Lauderdale, October 1994, sponsored by AACFS, NIH, CDC, and Univ. of Miami.

1994 - Chairperson of the Program Committee for the CFS Clinical Conference, held in Ft. Lauderdale, October 1994, sponsored by AACFS.

1991- 1997 Consultant to Center for Special Immunology, Inc., Ft. Lauderdale, FL.

1993 - 2000 - Board of Directors, American Association for Chronic Fatigue Syndrome.

1993- Present Medical Advisory Board, Chronic Fatigue and Immunodeficiency Syndrome Foundation.

1993- 1998 - Medical Advisory Board, Environmental Health Foundation.

2000 NIH State of the Science CFS Conference planning committee

2001 – present Name Change subcommittee, HHS CFS coordinating Committee

1999-present – CDC CFS Case Definition Revision Committee

2001 Canadian CFS Clinical Case definition expert panel

2001 CDC Expert Advisory Panel – long term outcomes study

2002 – present NIH reviewer and site visitor GCRC applications

2001- present Ad Hoc reviewer, Medical Research Council, United Kingdom.

2003 –present NIH reviewer CFS Special Emphasis Panel

2003 Brighton Collaboration on CFS Case Definition

2003 Elected to the Board of Directors, AACFS, 7 year term

2005 - 2009 President of the International Association for Chronic Fatigue Syndrome – this international organization of investigators and clinicians sponsors international and regional meetings, has developed a peer review journal, and works with government and regional groups to develop curricula and provider education programs.

2007 – 2011 CFSAC HHS Advisory committee to the Secretary of Health and Human Services

2010 – current Principal Proponent, National Gulf War Illness genomic bank and GWAS study, a VA cooperative study approved for planning, full protocol to be reviewed in Fall 2010.

Selected , recent and related publications (from 161 journal articles, 3 books, 20 invited chapters):

1. Jason LA, Unger ER, Dimitrakoff JD, Fagin AP, Houghton M, Cook DB, Marshall GD Jr, Klimas N, Snell C. Minimum data elements for research reports on CFS. Brain Behav Immun. 2012 Mar;26(3):401-6.

2. Klimas, NG, Broderick, G, Fletcher MA. Biomarkers for chronic fatigue. Brain Behav Immun 2012, in press.

3. Lattie EG, Antoni MH, Fletcher MA, Penedo F, Czaja S, Lopez C, Perdomo D, Sala A, Nair S, Fu SH, Klimas N.Stress management skills, neuroimmune processes and fatigue levels in persons with chronic fatigue syndrome. Brain Behav Immun. 2012 Mar 6.

4. Lopez C, Antoni M, Penedo F, Weiss D, Cruess S, Segotas MC, Helder L, Siegel S, Klimas N, Fletcher MA. A pilot study of cognitive behavioral stress management effects on stress, quality of life, and symptoms in persons with chronic fatigue syndrome. J Psychosom Res. 2011 Apr;70(4):328-34. Epub 2011 Jan 15.

5. Fletcher MA, Zeng XR, Maher K, Levis S, Hurwitz B, Antoni M, Broderick G, Klimas NG. Biomarkers in chronic fatigue syndrome: evaluation of natural killer cell function and dipeptidyl peptidase IV/CD26. PLoS One. 2010 May 25;5(5):e10817.PMID: 20520837

6. Fletcher MA, Rosenthal M, Antoni M, Ironson G, Zeng X, Barnes z, Harvey,J, Hurwitz B, Levis-Dusseau S, Broderick G, Klimas, N. Plasma neuropeptide Y: a biomarker for symptom severity in chronic fatigue syndrome. Behav Brain Funct 2010, 6:76.

7. Broderick G, Kreitz A, Fuite J, Fletcher MA, Vernon SD, Klimas N. A pilot study of immune network remodeling
under challenge in Gulf War Illness. Brain Behav Immun. 2011 25:302-313

8. Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. A formal analysis of cytokine networks in chronic fatigue syndrome. Brain Behav Immun. 2010 Oct;24(7):1209-17.

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10. Corina L, Antoni M, Penado F, Weiss D, Cruess S, Sagotas M-C, Helder L, Siegel, S, Klimas N, Fletcher MA. A pilot- study of cognitive behavioral stress management effects on stress, quality of life and symptoms in persons with chronic fatigue syndrome. J Psycho Res in press, 2011.

11. Fletcher MA, Zeng XR, Barnes Z, Lewis S, Klimas NG. Plasma cytokines in women with chronic fatigue syndrome. J Translational Med 2009, 7:96 (12 November 2009).
http://www.translationalmedicine.com/content/7/1/96

12. Whistler T, Fletcher MA, Lonergan W, Zeng XR, Lin JM, Laperriere A, Vernon SD, Klimas NG. Impaired immune function in Gulf War Illness. BMC Med Genomics. 2009 Mar 5;2:12.

13. Hurwitz BE, Coryell VT, Parker M, Martin P, Laperriere A, Klimas NG, Sfakianakis GN, Bilsker MS. Chronic fatigue syndrome: illness severity, sedentary lifestyle, blood volume and evidence of diminished cardiac function. ClinSci(Lond). 2009 May 26.

14. Fekete EM, Antoni MH, Lopez CR, Durán RE, Penedo FJ, Bandiera FC, Fletcher MA, Klimas N, Kumar M, Schneiderman N. Men's serostatus disclosure to parents: associations among social support, ethnicity, and disease status in men living with HIV. Brain Behav Immun. 2009 Jul;23(5):693-9. Epub 2009 Jan 21.

15. Greeson JM, Barry E. Hurwitz BE, Llabre MM, Schneiderman N, Penedo FJ, Klimas NG. Psychological Distress, Immune Regulation and Disease Severity in HIV/AIDS, Brain Behav Immun. 2008 22(6):901-11.

16. Klimas NG, Koneru AO Chronic fatigue syndrome: inflammation, immune function, and neuroendocrine interactions. Curr Rheumatol Rep. 2007 Dec;9(6):482-7.

17. Jones JF, Kohl KS, Ahmadipour N, Bleijenberg G, Buchwald D, Evengard B, Jason LA, Klimas NG, Lloyd A, McCleary K, Oleske JM, White PD; Brighton Collaboration, Fatigue Working Group. Fatigue: case definition and guidelines for collection, analysis, and presentation of immunization safety data. Vaccine. 2007 Aug 1;25(31):5685-96.

18. Siegel SD, Antoni MH, Fletcher MA, Maher K, Segota MC, Klimas N. Impaired natural immunity, cognitive dysfunction, and physical symptoms in patients with chronic fatigue syndrome: preliminary evidence for a subgroup? Psychosom Res. 2006 Jun;60(6):559-66.

19. Craddock RC, Taylor R, Broderick G, Whistler T, Klimas N, Unger ER.Exploration of statistical dependence between illness parameters using the entropy correlation coefficient. Pharmacogenomics. 2006 7(3):421-8.

20. Broderick G, Craddock RC, Whistler T, Taylor R, Klimas N, Unger ER, Identifying illness parameters in fatiguing syndromes using classical projection methods. Pharmacogenomics. 2006 Apr;7(3):407-19.

21. Whistler T, Taylor R, Craddock RC, Broderick G, Klimas N, Unger ER. Gene expression correlates of unexplained fatigue. Pharmacogenomics. 2006 Apr;7(3):395-405.

Funded research (last five years)

Active

Study of Chronic Fatigue Syndrome using comprehensive molecular profiling with network and control theory
NIAMSD 1 R01 AR057853-01A1 (PI NG Klimas) 11/1/2010 – 10/31/2014
The objective of this study is to improve our understanding of CFS pathogenesis by using broad-scale molecular profiling to create a comprehensive assessment of status in several of the body's regulatory systems
Role: PI

Immunologic Mechanisms, Biomarkers and Subsets in CFS
NIAID R01AI065723 (PI MA Fletcher) 12/1/06 – 11/30/12
Goal of this project is to determine the immunologic basis for CFS pathogenesis
Role: coPI, 25% effort
Virtual platforms for CBT in CFS/ME
NIH NIMH RO1 (PI Mike Antoni) 2010 – 2014 ,
A study of spouse and patient CBT using a virtual platform.
Role: coPI

The Use of Comprehensive Molecular Profiling with Network and Control in GWI
GW080152 DOD (PI N Klimas) 07/15/2009 - 06/14/2012
GWI patients will be studied using gene array technology and neuron-endocrine-immunologic profiling pre-post exercise challenge.
Role: PI, 20% effort

Theory-Driven Models for Correcting "Fight or Flight" Imbalance in Gulf War Illness
GW093042 DOD (PI G Broderick) 08/01/2010 – 07/30/2013
A computer-based analysis of the hypothalamic-pituitary-adrenal (HPA) axis, the immune system, and the ability of those systems to ensure survival and then to re-establish homeostasis that existed prior to the "fight or flight" stimulus.
Role: co-I, 05% effort

A Clinical and Biosample Database to Enable Discovery of Pathogens and Pathogenic Mechanisms in Chronic Fatigue Syndrome,
CFI (Chronic Fatigue Initiative) 2/2011- 2/2013
Role: PI Funding approx $1,400,000 over 2 years.
DOD : Consortium 1 year funding to develop GWI translational research program,
role CoPI, with Wayne State University , Marianna Morris; neurotoxicology theme
DOD: Consortium 1 year funding to develop GWI research team studying neuroinflammation in GWI, Kim Sullivan, Boston University PI, role co-I

CDC Contract: Developing a clinical data base for longitudinal studies in CFS
Role site PI, coinvestigator
$130,000/year Start date May 2012
NIAID RO1
"The role of XMRV in CFS" PI, Ian Lipkin, Columbia University
Role: National Clinical Core Coordinator and Miami site PI 2 year study local budget $125,000

Completed (last 5 years)
Neuropeptide Y and dipeptidyl-peptidase IV (CD26) in chronic fatigue syndrome
NIAAA R21AA016635 (PI MA Fletcher) 9/30/06-5/31/09
Goal of this project was to determine the relationship of neuropeptide Y and dipeptidylpeptidase IV to natural killer cell cytotoxicity in CFS.
Role: coPI
Psychobiological Processes and Health in HIV.
NIMH R01 (PI G. Ironson) 7/01/03-5/31/09
This grant examined psychological and biological (CTL, NK, cortisol) predictors of disease progression in HIV/AIDS.
Role: CoI.

Patterns of Gene Activation in Gulf War Illness and Chronic Fatigue Syndrome
VA Merit Review, (PI N Klimas). 11/04 -11/09
Male and Female GWI and CFS patients were studied using gene array technology pre-post exercise challenge.
Role: PI

Efficacy of an Emotional Exposure Intervention in HIV.
NCCAM R01 (PI G. Ironson) 7/01/03-6/30/08
This study investigated the efficacy of emotional exposure and depth processing though writing on well being in patients with HIV/AIDS.
Role: Co.I. 5% effort

HIV/HCV Co-infection: HAART and Pathophysiology
NIHLBI R01 (PI B Hurwitz) 7/31/04 to 8/31/07
This study looked at the interactions of HAART and co-infection with HCV on the pathophysiology of HIV/AIDS
Role: Co-I, 5% effort

Massage Benefits in HIV+ Children: Mechanisms of Action
NCCAM R01AT002689 (PI G. Shor-Posner) 01/01/05-03/31/07
The two-fold intent of the above proposal was to evaluate the impact of massage therapy on immune recovery, and to investigate a potential neuroimmune mechanism of massage action.

APPENDIX G: Gordon Broderick, Ph.D. Curriculum Vitae

CONTACT INFORMATION

Home.

Office. Division of Pulmonary Medicine, Department of Medicine
University of Alberta, Suite 225B College Plaza
8215 112 Street NW, Edmonton, Alberta, Canada T6G 2C8
Ph. 780-445-4666 Fx. 780-407-6384 Email. gordon.broderick@ualberta.ca

Citizenship: Canadian Languages: French and English both spoken and written fluently

EDUCATION AND TRAINING

Ph.D. Chemical Engineering	École Polytechnique de Montréal	1991-1994
Masters Chemical Engineering	McGill University	1988-1989
Bachelor Mechanical Engineering	McGill University	1980-1984

LICENSES.

Ing. (P. Eng.)	Ordre des Ingénieurs du Québec	1986-present

HONORS AND AWARDS

Nightingale Award for Community Service, ME Society of Edmonton	2010
Associate Editor BMC Systems Biology	2010
Invited member US Veteran's Administration Committee on Gulf War Illness	2010
Teacher of the Year Award (Small Group Case Study)	2008
Dean of Graduate Studies and Research Award for best doctoral thesis.	1994
Natural Sciences and Engineering Research Council of Canada Award (Ph.D)	1991 - 1994
John S. Bates Centennial Fellowship (Ph.D)	1992 - 1993
R.M. Fowler Memorial Fellowship (Masters)	1988 - 1989
Natural Sciences and Engineering Research Council of Canada Award (Masters)	1988 - 1989
J.B. Lynch Foundation Award (Bachelor)	1980 - 1984

PROFESSIONAL /ACADEMIC POSITIONS HELD

Associate Professor – Dept. of Medicine, July 1, 2006 – Present
Faculty of Medicine and Dentistry, University of Alberta • Edmonton, AB
Reporting to the Department Chair, establish independent research program in the area of computational medicine. **Major Contributions**: (1) Principal investigator in computational research effort directed at understanding immune cell activation and population kinetics in chronic inflammatory illnesses (2) Actively developing agent-based model of the spatial dynamics of immune cell migration and signalling in the context of neuro-immune dysfunction. (3) Co-investigator /computational group leader in $12M project directed at understanding gene regulatory mechanisms involved in injury and alloimmune response of kidney grafts.

Project Leader – Cell Simulation 2002 – 2006
Institute for Biomolecular Design – University of Alberta • Edmonton, AB
Reporting to the Executive Director, direct the research activities of an interdisciplinary group of 4-6 programmers and engineers working to create spatially discrete mathematical models of cell life.

Major Contributions: (1) Lead architect in creation of scalable agent-based high-performance computational framework for whole cell simulation, (2) Developed scientific strategy behind $9.5M IBM sponsorship of dedicated high-performance computing platform, (3)Developed first agent-based model of cell membrane chemistry / mechanics, (4) 2 U.S. Provisional Patents, 2 PCTs in 18 months.

Consulting Biostatistician – Transplant Transcriptome Project **2004 – 2005**
Alberta Transplant Applied Genomics Centre – University of Alberta • Edmonton, AB
Reporting to the Executive Director, assist in the development of a strategy for ensuring the use of leading edge data analysis tools in supporting the research activities. **Major Contributions**: (1) Outlined standardised data handling and analysis pipeline, (2) Designed multivariate calibration filter for reconciliation of new micro-array architecture with previous generation, (3) Proposed strategy for establishing future biostatistics team, identifying key internal and external contributors.

Principal Scientist **1999 – 2002**
Noranda Technology Center • Pointe-Claire, QC
Reporting to the Senior Vice-President Technology, formulate company-wide plan for developing strategic technologies in advanced process systems. Direct research efforts of project leaders in this area. Principal investigator with substantial discretion, define and execute high-impact research. **Major Contributions**: (1) Developed novel Monte Carlo signal processing technique for estimating particle size distributions from laser pulse patterns, (2) Lead research collaboration with McGill University directed at modelling complex spatial distributions using discrete stochastic automata, (3) Lead high-risk collaboration with University of Lund aimed at innovative applications of artificial reasoning (reinforcement learning) for advanced diagnostics.

Visiting Scientist **2000 – 2001**
McGill School of Computer Science / Montreal General Hospital • Montreal, QC.
Responsible for computational aspects of multidisciplinary effort directed at elucidating pathway kinetics and specificity of steroid-containing drugs in treating immune disorders using gene expression time course sequences. **Major Contributions**: (1) Completed n-way projection analysis of multi-tissue time-course responses to steroid impulse stimuli, (2) Implemented and evaluated algorithm for multivariate linear filtering, discovery and class prediction for AML and ALL-type leukemia, (3) Conducted comparison of results from PCA and self-organising maps (SOM) for extraction of features from gene expression in yeast cell cycle data.

Senior Scientist **1995 – 1999**
Noranda Technology Center • Pointe-Claire, QC
Initiate and actively lead new research projects using discretionary funding to identify promising new technologies, develop proof of concept. Effectively coordinate academic and private consultants. Appointed by Senior Vice-President Technology to committee reviewing Noranda's high-impact research initiatives. **Major Contributions**: (1) Patented innovative energy-saving refining strategy. (2) Established partnership with Colorado State University, and Hong Kong University in evolutionary programming. (3) Established research partnership with University of Waterloo for wavelet-based adaptive estimation and control. (4) Defined, and obtained funding for a 2-year collaborative research with McGill University on cellular automata (CA).

Research Engineer/Project Leader **1989 - 1995**
Noranda Technology Center • Pointe-Claire, QC
Optimize process performance by applying mathematical modeling and simulation techniques. Develop and maintain close ties with academic institutions and other private research facilities to help identify promising new technologies, transfer, and adapt these to meet strategic needs. Act as a technical leader in statistical

process modeling. **Major Contributions**: (1) Developed detailed product quality models based on intrinsic material properties. (2) Constructed novel multivariate statistical approach to quality monitoring. (3) Successfully implemented PLS model-based system for on-line prediction and quality optimization.

PERSONAL STATEMENT

A biochemical and systems engineer by training, I bring over 15 years interdisciplinary research experience in analysis of complex data sets, predictive modeling, and numerical optimization. My current research is focused on understanding immune dysfunction and autoimmunity from an integrated systems perspective. In particular my group is investigating how imbalances between the immune system's multiple components and its interactions with the endocrine and nervous systems may lead to complex disorders like Gulf War Illness and myalgic encephalomyelitis/ chronic fatigue syndrome (ME/CFS). Moving beyond a conventional one piece at a time approach, we are using information and dynamic systems theory to identify altered structure and function in endocrine-immune networks. This is leading us to specific pathways that underlie pathogenic immune conversations and we are using our experience in computational biochemistry (leader Project CyberCell) to conduct detailed investigations of limiting step reactions. Importantly, these models serve to simulate strategies for re-directing immune and endocrine processes using well-chosen sequence of interventions. This research has attracted funding from the US Department of Defense (2 Investigator Initiated awards), the NIH (co-PI on R01) as well as national associations in the US. With an established track record of collaboration with prominent US
clinical and basic scientists in Miami, Chicago and Atlanta, I have served on two NIH study sections for CFS research, a VA Cooperative Studies Program planning committee and been called upon to present my research to the VA Research Advisory Committee. This research experience at the intersection of the computational and life sciences is supportive of my role as principal investigator on this application.

B. POSITIONS AND HONORS

Positions

1989 - 95 Research Project Leader, Process control and optimization group, Noranda Technology Center, Pointe-Claire, Quebec, Canada
1995 - 99 Senior Scientist, Noranda Technology Center • Pointe-Claire, Quebec, Canada
1995 Adjunct Professor, Dept. Applied Mathematics and Industrial Engineering, École Polytechnique de Montréal, Montreal,Quebec, Canada
1999-02: Principal Scientist, Noranda Technology Center • Pointe-Claire, Quebec, Canada
2000-01 Visiting Scientist, McGill School of Computer Science / Montreal General Hospital, Montreal, Quebec, Canada.
2002–06 Project Leader – Cell Simulation, Institute for Biomolecular Design – University of Alberta, Edmonton, Alberta, Canada
Principal Investigator/Program Director (Last, First, Middle): Broderick, Gordon2
2006 - present Associate Professor, Dept. of Medicine, Faculty of Medicine and Dentistry, University of Alberta, Alberta, Canada
2010 - present Associate Editor, BMC Systems Biology (Impact 4.06), London, UK.
2011 - present Voluntary Associate Professor, Dept. of Medicine, Miller School of Medicine, University of Miami, Miami, FL

Awards and Recognition

2010 Discovery Learning Preceptor Excellence Award, Faculty of Medicine, University of Alberta
2010 Nightingale Award for service to the patient community, M.E./C.F.S. Society of Edmonton
2008 Teacher of the Year Award (Small Group Study), Faculty of Medicine, University of Alberta
1994 Dean of Graduate Studies and Research Award for best doctoral thesis.
1991 – 94 Natural Sciences and Engineering Research Council of Canada Award (Ph.D)
1992 – 93 John S. Bates Centennial Fellowship (Ph.D)

1988 – 89 R.M. Fowler Memorial Fellowship (Masters)
1988 – 89 Natural Sciences and Engineering Research Council of Canada Award (Masters)
1980 – 84 J.B. Lynch Foundation Award (Bachelor)

C. PEER-REVIEWED PUBLICATIONS (FROM 39 PEER REVIEWED ARTICLES)

Peer-reviewed Journals. Separated into major areas of contribution.

 Contributions to the study of networked regulatory systems in complex illness. Novel contributions were made to the quantitative analysis of complex biological networks revealing i) that CFS manifests altered patterns of hormone-neurotransmitter-gene interaction across multiple regulatory systems, and ii) that this coincides with a distinct mode of operation available to this regulatory axis by virtue of its complexity. We demonstrated that it is theoretically plausible to design an intervention capable of resetting the endocrineimmune axis and restoring stable function. These findings were publicized in local and national media.

1. Folcik VA, Broderick G, Mohan S, Block B, Ekbote C, Doolittle J, Khoury M, Davis L, Marsh CB. Using an agent-based model to analyze the dynamic communication network of the immune response. Theor Biol Med Model. 2011 Jan 19;8(1):1. [Epub ahead of print]

2. Broderick G, Kreitz A, Fuite J, Fletcher MA, Vernon SD, Klimas N. A pilot study of immune network remodeling under challenge in Gulf War Illness. Brain Behav Immun. 2011 Feb; 25(2): 302-13.

3. Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. A formal analysis of cytokine networks in chronic fatigue syndrome. Brain Behav Immun. 2010 Oct; 24(7): 1209-17.

4. Ben-Zvi A, Vernon SD, Broderick G. 2008. Model-based Therapeutic Correction of Hypothalamic Pituitary Adrenal Axis Dysfunction. PLoS Comput Biol 5(1): e1000273. doi:10.1371/journal.pcbi.1000273.

5. Aspler AL, Bolshin C, Vernon SD, Broderick G. 2008. Evidence of Inflammatory Immune Signaling in Chronic Fatigue Syndrome: A Pilot Study of Gene Expression in Peripheral Blood. Behav Brain Funct Sep 26;4(1):44. doi:10.1186/1744-9081-4-44.

6. Fuite J,Vernon SD, Broderick G. 2008. Neuroendocrine and Immune Network Re-modeling in Chronic Fatigue Syndrome: An Exploratory Analysis. Invited submission, Genomics 92(6): 393-399. doi:10.1016/j.ygeno.2008.08.008 (issue cover).

7. Craddock RC, Taylor R, Broderick G, Whistler T, Klimas N, Unger ER, 2006. Exploration of statistical dependence between illness parameters using the Entropy Correlation Coefficient. Pharmacogenomics 7(3): 421-428.

 Contributions to the study of molecular phenotyping of disease and computational genomics. Leading contributions were made in the following work towards advancing the definition disease phenotypes at molecular level resolution. In particular novel applications of hierarchical and time course variants of statistical projection models were deployed towards the novel description of CFS patients and graft rejection patterns in kidney transplantation. In both cases this represented a significant advance in diagnostic ability over the conventional clinical or histopathological assessment.

8. Fletcher MA, Rosenthal M, Antoni M, Ironson G, Zeng XR, Barnes Z, Harvey JM, Hurwitz B, Levis S, Broderick G, Klimas NG. Plasma neuropeptide Y: a biomarker for symptom severity in chronic fatigue syndrome. Behav Brain Funct. 2010 Dec 29;6:76.

9. Fletcher MA, Zeng XR, Maher K, Levis S, Barry H, Antoni M, Broderick G, Klimas NG. 2010. Biomarkers in chronic fatigue syndrome: Evaluation of natural killer cell function and dipeptyl

peptidase IV. PLoS ONE. 25 May | 10.1371/journal.pone.0010817.

10. Nakamura T, Schwander SK, Donnelly R, Ortega F, Togo F, Broderick G, Yamamoto Y, Cherniack NS, Rapoport D, Natelson BH. 2010. Cytokines across the night in chronic fatigue syndrome with and without Fibromyalgia. Clin Vaccine Immunol. Feb 24. [Epub ahead of print]

11. Mueller TF, Einecke G, Reeve J, Sis B, Mengel M, Jhangri G, Bunnag S, Cruz, J, Wishart D, Meng C, Broderick G, Kaplan B, Halloran PF, 2007. Microarray analysis of rejection in human kidney transplants using pathogenesis-based transcripts sets. Am J Transplant 7(12): 2712-2722.

12. Famulski KS, Broderick G, Einecke G, Hayl K, Cruz J, Sis B, Mengel M Halloran PF. 2007. Transcriptome analysis reveals heterogeneity in the injury response of kidney transplants. Am J Transplant 7: 1–13.

13. Einecke G, Broderick G, Sis B, Halloran PF, 2007. Early loss of renal transcripts in kidney allografts: relationship to morphologic changes and alloimmune effector mechanisms. Am J Transplant, 7(5): 1121–1130.

14. Broderick G, Craddock RC, Whistler T, Taylor R, Klimas N, Unger ER, 2006. Identifying illness parameters in fatigue syndromes using classical projection methods. Pharmacogenomics 7 (3): 407-419.

15. Whistler T, Craddock RC, Taylor R, Broderick G, Klimas N, Unger ER, 2006. Gene expression correlates of fatigue. Pharmacogenomics 7(3): 395-405.
 Contributions to the study of complex intracellular reaction kinetics and virtual life. These works describe leadership in the large-scale computational modeling of cellular life at the molecular level. Particle based simulations unparalleled in scale at the time were developed and used to study cell metabolism under conditions of intracellular crowding and low enzyme copy number as well as the emergent properties of cell membranes. This lead to invited lectures at institutions including the Weizmann Institute and the Center for Complexity Studies in Israel. The work was also publicized in the magazine *The Scientist*.

16. Zhao J, Ridgway D, Broderick G, Kovalenko A, Ellison M, 2008. Extraction of elementary rate constants from global network analysis of E. coli central metabolism. BMC Systems Biology 2:41doi:10.1186/1752-0509-2-41.

17. Ridgway D, Broderick G, Ru'aini M, Winter P, Lopez-Campistrous A, Ellison MJ. 2008. Coarsegrained molecular simulation of diffusion and reaction kinetics in a crowded virtual cytoplasm. Biophys J May 15; 94(10):3748-59.

18. Broderick G, Rubin E, 2006. The Realistic Modelling of Biological Systems: A Workshop Synopsis. ComPlexUs 3:217–230.

19. Ridgway D, Broderick G, Ellison MJ, 2006. Accommodating space, time and randomness in network simulation. Invited paper. Curr Opin Biotechnol 17:1-6.

20. Broderick G, Ru'aini M, Chan E, Ellison MJ, 2004. A Life-like Virtual Cell Membrane Using Discrete Automata. Invited Paper, In Silico Biology 5: 0016.

Active Funded Research (Last five years)

Molecular Patterns of Persistent Immune Activation in a Post-infectious Adolescent Cohort (PI)
Supporting agency: **The CFIDS Association of America**
Performance period: March 1, 2009 – May 15, 2011
Level of funding: $125,000 USD
Project goals. To describe deviations in endocrine-immune status and signaling that occur with onset and progression of post-infectious chronic fatigue syndrome (PI-CFS) for early detection and objective diagnosis.
Role and responsibilities: As PI assemble and lead a cross-disciplinary team from 4 institutions to capitalize on a large PI-CFS cohort, already recruited and clinically assessed over a 2-year period as well as ongoing systems biology research.

The Use of Comprehensive Molecular Profiling with Network and Control Theory to Better Understand Gulf War Illness and Model Therapeutic Strategies (Co-PI)
Supporting agency: **Congressionally Directed Medical Research Program (CDMRP) /DOD Gulf War Illness Research Program Investigator-initiated Research Award**
Performance period: June 15, 2009 – June 14, 2011 (extended to June 2012)
Level of funding: $164,000 USD sub-award to University of Alberta
Project goals. We propose that GWI and CFS result not only from failure of individual neuroendocrine and immune components but also from a significant deterioration of their regulatory interaction as a result of infectious, environmental or psychological insult. This study aims to verify this hypothesis by monitoring levels of immune and neuroendocrine markers in blood prior, during and after an exercise challenge.
Role and responsibilities: As Co-PI lead the computational component of the project namely the identification and interpretation of network models describing immune and endocrine regulation in these subjects.

Theory-driven Models for Correcting "Fight or Flight" Imbalance in Gulf War Illness. (PI)
Supporting agency: **Congressionally Directed Medical Research Program (CDMRP) /DOD Gulf War Illness Research Program Investigator-initiated Research Award**
Performance period: July 1, 2010 – June 31, 2013
Level of funding: $679,000 USD sub-award
Project goals. The goal of this project is Create a comprehensive engineering model of endocrine-immune interaction dynamics in order to identify (i) theoretical failure modes of the HPA-immune axis that align with GWI, and (ii) promising treatment strategies that exploit the regulatory dynamics of these systems to reset control of the HPA-immune axis to normal.
Role and responsibilities: As PI, assemble a cross-disciplinary team and lead the project.

Study of Chronic Fatigue Syndrome using comprehensive molecular profiling with network and control theory.
Supporting agency: **National Institutes of Health (NIH) (R01).**
Performance period: October 1, 2010 – November 1, 2014
Level of funding: $440,000 USD
Project goals. To improve our understanding of CFS pathogenesis by: (i) integrating data and knowledge collected from disparate sources across several of the body's regulatory systems, (ii) mapping the interactions that emerge at multiple scales of biology and identifying potentially altered "wiring" in these signaling networks specific rapid response to exercise in CFS.
Role and responsibilities: As Co-PI lead the computational component of the project namely the identification and analysis of network models describing endocrine-immune regulation in these subjects

Appendix H: Mary Ann Fletcher Curriculum Vitae

UNIVERSITY OF MIAMI
Curriculum Vitae

Date: January 28, 2012

I. PERSONAL

1. Name: Mary Ann Fletcher
2. Home Phone:
3. Office Phone: 305243-6288
4. Home Address:
5. Current Academic Rank: Professor, tenured
6. Primary Department: Medicine
7. Secondary Appointments: Microbiology/Immunology; Psychology; Pediatrics, member of the Graduate Faculty
8. Citizenship: USA

II. HIGHER EDUCATION

1. Texas Technological College, B.S. (honors), 1959
2. University of Texas, M.A., 1961
3. Baylor University, Ph.D., 1966
4. Northwestern University, postdoctoral fellowship, 1966-68

III. CERTIFICATIONS AND LICENSURES

1. Diplomat American Board of Bioanalysis - High Complexity Laboratory Director, Clinical & Technical Consultant
2. State of Florida licensed and CLIA certified as Clinical Laboratory Director

IV. EXPERIEINCE

1. 1981 - Present: Tenured Professor of Medicine, University of Miami Miller School of Medicine, Miami, FL
2. 1982 - Present: Professor of Microbiology/Immunology, UM Miami School of Medicine, Miami, FL
3. 1989 - Present: Professor of Psychology, UM, Coral Gables, FL
4. 1982 - Present: Director, E.M. Papper Clinical Immunology Laboratory, UM School of Medicine, Miami, FL
5. 1978 - Present: Member of the Graduate Faculty, UM, Coral Gables, FL
6. 1978 -1981: Associate Professor of Microbiology, UM School of Medicine, Miami, FL
7. 1977 - 1981: Tenured Associate Professor of Medicine, UM School of Medicine, Miami, FL
8. 1972 – 1976 : Assistant Professor of Medicine, UM School of Medicine, Miami, FL
9. 1970 - 1972: Adjunct Assistant Professor of Biology, Illinois Institute of Technology, Chicago, IL
10. 1969 -1972: Assistant Director, Division of Hematology, Michael Reese Hospital and Medical Center, Chicago, IL
11. 1969 -1972: Assistant Attending Physician, Special Staff, Michael Reese Hospital and Medical Center, Chicago, IL..

12. 1967 -1969: Instructor and Asst Professor, Dept. Microbiology, Northwestern University Medical School, Chicago, IL
13. 1966 - 1969: Postdoctoral fellow, Northwestern University, Evanston Hospital, Evanston, IL
14. 1963 - 1966: Research Associate, Graduate Research Institute of Baylor University, Dallas, TX
15. 1962 - 1963: Clinical Bacteriologist, Spohn Hospital & Driscoll Found. Hospital, Corpus Christi, TX
16. 1959 - 1961: Research Assistant, Dept Microbiology, Univ. of Texas, Southwestern Medical School, Dallas, TX.

Personal Statement

I have completed NIH-, VA- and DOD-funded research on GWI and CFS/ME and other complex multi symptom illnesses, including cancer and HIV for many years. My work has a particular focus on identification of clinically useful biomarkers. I have over 35 years experience in clinical laboratory immunology and 29 years as a senior scientist in interdisciplinary research. I am currently the Director of the E.M. Papper Clinical Immunology Laboratory at the University of Miami Miller School of Medicine, approved by Medicare and Medicaid under CLIA '88 as a laboratory director for high complexity assays. I am expert in laboratory quality control and immune laboratory methodology, including flow cytometry, measurement of lymphocyte function, and determination of soluble immune biomarkers by ELISA and RIA. I am familiar with safety and quality assurance procedures and methods to minimize pre-analytical, analytical and post-analytical variability. I have published 259 peer reviewed articles and invited chapters.

Positions and Honors

Positions and Employment

1968–1969 Assistant Professor, Dept. of Microbiology, Northwestern Univ. Medical School, Chicago, IL
1970–1972 Assistant Director, Division of Hematology, Michael Reese Hospital, Chicago, IL
1972–1981 Asst & Assoc Professor of Medicine, Div. of Immunology, U. of Miami Sch. of Med., Miami, FL.
1980–Present Director, E.M. Papper Laboratory of Clinical Immunology.
1981–Present Professor of Medicine (tenured), Microbiology/Immunology and Psychology, Miller School of Medicine.

Licensure

1984–Present Licensed as a Clinical Laboratory Director in Florida

Peer Review Committees and Consulting

1980 – 1982 NCI, Tumor Immunology Review Committee
1978 – 1984 NCI - Clinical Cancer Program Project Review Committee Site Visit Participant
1979 – 1980 Member, NSF, Postdoctoral Fellowship Review Committee
1984 – 1987 NIH, Immunotechnology Special Review Committee, Ad Hoc reviewer for AIDS study section
1991 NIMH Site Visitor,
1996 NIDA. Site Visitor
1998 NHLBI, Site Visitor, special review committee
2002 - 2010 NIAID, CFAR special review committee, HIV vaccine development review committee, DAIDS special emphasis panel
2008, 2010 NIH, CFS special review panel
2011 - present FDA, consultant on medical devices and diagnostics
2012 member of the HHS CFS Advisory Committee
2012 NIH Common Fund's Single Cell Analysis program initiative, special review panel

Honors

Fellow, Academy for Behavioral Medicine Research
Diplomate, American Board of Bioanalysis

Excellence in Research Award, IACFS Ottawa, 2011.

Publications (past three years and others relevant to the current application selected from 259 peer reviewed articles and invited chapters)

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FUNDED RESEARCH (LAST FIVE YEARS)

Active

Immunologic Mechanisms, Biomarkers and Subsets in CFS NIAID
R01AI065723 (PI MA Fletcher) 12/1/06 – 11/30/12
Goal of this project is to determine the immunologic basis for CFS pathogenesis
Role: PI, 25% effort

The Use of Comprehensive Molecular Profiling with Network and Control in GWI
GW080152 DOD (PI N Klimas)
07/15/2009 - 06/14/2012
GWI patients will be studied using gene array technology and neuron-endocrine-immunologic profiling pre-post exercise challenge.
Role: co-PI, 20% effort

Theory-Driven Models for Correcting "Fight or Flight" Imbalance in Gulf War Illness
GW093042 DOD (PI G Broderick) 08/01/2010 – 07/30/2013
A computer-based analysis of the hypothalamic-pituitary-adrenal (HPA) axis, the immune system, and the ability of those systems to ensure survival and then to re-establish homeostasis that existed prior to the "fight or flight" stimulus.
Role: co-I, 05% effort

Study of Chronic Fatigue Syndrome using comprehensive molecular profiling with network and control theory
NIAMSD 1 R01 AR057853-01A1 (PI NG Klimas) 11/1/2010 – 10/31/2014
The objective of this study is to improve our understanding of CFS pathogenesis by using broad-scale molecular profiling to create a comprehensive assessment of status in several of the body's regulatory systems
Role: CoPI, 30% effort

Virtual platforms for CBT in CFS/ME
NIH NIMH RO1 (PI Mike Antoni) 2010 – 2014 ,
A study of spouse and patient CBT using a virtual platform.
Role: CoPI

Completed (last 5 years)

Neuropeptide Y and dipeptidyl-peptidase IV (CD26) in chronic fatigue syndrome
NIAAA R21AA016635 (PI MA Fletcher) 9/30/06-5/31/09
Goal of this project was to determine the relationship of neuropeptide Y and dipeptidyl- peptidase IV to natural killer cell cytotoxicity in CFS.
Role: PI, 20% effort

Psychobiological Processes and Health in HIV. NIMH R01
(PI G. Ironson) 7/01/03-5/31/09
This grant examined psychological and biological (CTL, NK, cortisol) predictors of disease progression in HIV/AIDS.
Role: CoI. 5% effort

Patterns of Gene Activation in Gulf War Illness and Chronic Fatigue Syndrome
VA Merit Review, (PI N Klimas). 11/04 -11/09
Male and Female GWI and CFS patients were studied using gene array technology pre-post exercise challenge.
Role: Co-PI, 20% effort

Efficacy of an Emotional Exposure Intervention in HIV. NCCAM
R01 (PI G. Ironson) 7/01/03-6/30/08
This study investigated the efficacy of emotional
exposure and depth processing though writing on
well being in patients with HIV/AIDS. Role: Co.I.
5% effort HIV/HCV Co-infection: HAART
andPathophysiology
NIHLBI R01 (PI B Hurwitz) 7/31/04 to 8/31/07
This study looked at the interactions of HAART and co-infection with HCV on the
pathophysiology of HIV/AIDS
Role: Co-I, 5% effort

Massage Benefits in HIV+ Children: Mechanisms of Action
NCCAM R01AT002689 (PI G. Shor-Posner) 01/01/05-03/31/07
The two-fold intent of the above proposal was to evaluate the impact of massage therapy on immune recovery, and to investigate a potential neuroimmune mechanism of massage action.
Role: Co.I. 10% effort

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Biomarkers for chronic fatigue

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Abstract

Fatigue that persists for 6 months or more is termed chronic fatigue. Chronic fatigue (CF) in combination with a minimum of 4 of 8 symptoms and the absence of diseases that could explain these symptoms, constitute the case definition for chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME). Inflammation, immune system activation, autonomic dysfunction, impaired functioning in the hypothalamic-pituitary-adrenal axis, and neuroendocrine dysregulation have all been suggested as root causes of fatigue. The identification of objective markers consistently associated with CFS/ME is an important goal in relation to diagnosis and treatment, as the current case definitions are based entirely on physical signs and symptoms. This review is focused on the recent literature related to biomarkers for fatigue associated with CFS/ME and, for comparison, those associated with other diseases. These markers are distributed across several of the body's core regulatory systems. A complex construct of

symptoms emerges from alterations and/or dysfunctions in the nervous, endocrine and immune systems. We propose that new insight will depend on our ability to develop and deploy an integrative profiling of CFS/ME pathogenesis at the molecular level. Until such a molecular signature is obtained efforts to develop effective treatments will continue to be severely limited.

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1. Background

Fatigue is a common symptom that includes both physical and mental components. It can be an acute response to physical, mental or infectious triggers and usually decreases as the trigger recedes. It can occur in healthy people as a result of physical and/or mental exertion. Symptoms of fatigue persisting for 6 months or more define chronic fatigue (CF). A survey of 2323 residents of Norway by Loge et al. (1998)reported CF to be endorsed by 11% of the general population. Of those respondents reporting no known disease or current heath problem, 7% had CF. In contrast, in Japan, more than half of the general adult population complained of fatigue, and more than one third of the population endorsed CF (Watanabe et al., 2008).

Disease associated fatigue may be directly related to the disease mechanisms (primary fatigue) or may be secondary to non-disease-specific factors. Measurement of fatigue can be from either the subjective or the objective standpoint and includes physical fatigue, reduced motivation, reduced activity, and mental fatigue. The magnitude and scope of debilitating fatigue is a central component in health care where chronic illness is a growing concern. Current acute illness research models are inadequate for resolving chronic disorders affecting multiple regulatory systems and presenting with complex constellations of symptoms. Fatigue is a fundamental component in a diverse array of illnesses that affect a broad patient demographic.

There is a growing body of research directed toward understanding the biology of fatigue; this research leads us to regard fatigue as a complex construct of symptoms that emerges from alteration and/or dysfunction in the nervous, endocrine and immune systems. Identifying biomarkers of CF is an important part of this effort. The official NIH definition of a biomarker is: “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” (Biomarkers Definitions Working Group, 2001). In this review of recent work, we selected examples of research on CF occurring in disease with an emphasis on chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME). The biomarker research examined included several levels of biology ranging from the intracellular (transcript) to the inter-cellular (cytokine and hormone) and behavioral levels (clinical and psychometric measures). Our aim in the selection of studies was not to be inclusive, but rather to include a representative sample of recent CF studies. Most

commonly, CFS/ME biology and that of other illnesses has focused on the detailed characterization of individual components taken in isolation. It is now clear that further understanding of disease mechanisms will require more than a list of the abundance of gene products, proteins or cells. These various cellular and molecular components are highly inter-related. Depending on the context various biological networks will become active to ensure appropriate regulatory feedback for maintaining homeostasis. In a review of CFS/ME, published in Lancet, Prins et al. (2006) stated: “Techniques such as bioimaging and proteomic strategies, and perhaps a systems biology approach, should be applied to try to elucidate such complicated interactions”. Advances in multiplex laboratory tests and bioinformatics allow fatigue research to advance beyond individual biomarkers to modular patterns of co-expression.

2. Biomarkers associated with fatigue in patients with identified diseases or syndromes other than CFS/ME

In a recent review, Graff et al. (2011) noted reports of moderate to severe fatigue in 50–70% of the cases in immune-mediated inflammatory diseases. As reported by the New York Times (Crouse, 2011) following her withdrawal August 31, 2011 from the US Open tennis tournament, Venus Williams, winner of seven Grand Slam singles titles, said, “The fatigue is hard to explain unless you have it. Some mornings I feel really sick, like when you don’t get enough sleep or you have flu or a cold. I always have some level of tiredness. And the more I tried to push through it, the tougher it got.” Williams received the diagnosis of the autoimmune disorder, Sjögren’s syndrome, a condition correlated with CF, earlier in the month. This CF is similar to “sickness behavior” in animals which is mediated by pro-inflammatory cytokines, in particular interleukin (IL)-1, acting on neuronal brain cells (Dantzer, 2001). Increased levels of IL-1 receptor antagonist (IL-1Ra) in the cerebrospinal fluid were associated with increasing fatigue in primary Sjögren’s syndrome patients, supporting the suggestion that the IL-1 system is a possible biomarker of fatigue (Harboe et al., 2009) (Table 1).

Table 1. Biomarkers associated with fatigue in patients with identified diseases or syndromes (other than CFS/ME) and selected from recent studies.

Disease associated with fatigue	Biomarker associated with fatigue	Reference
Sjögren's syndrome	>IL-1Ra in CSF	Harboe et al, 2009
Rheumatoid arthritis	>Stimulated production of IL-6	Davis et al, 2008
	Depression & anxiety	Stebbings, et al, 2010
	Absence of RF; >DAS-28; >pain	van Hoogmoed, et al, 2010
Systemic lupus erythematosus	>Serum IFNα	Lee et al, 2010
	>CRP	Rezaieyazdi, et al, 2011
Inflammatory bowel disease	>CRP	Graff et al, 2011
	Hemoglobin; GI symptoms; altered sleep	Jesness-Jorgensen, 2011
Multiple sclerosis	>Frequency of TNFα & IFNγ producing CD8+ T cells	Gold, et al, 2011
	>TNFα mRNA in T cells	Flachenecker et al, 2006
	Post exercise >β-1 & β-2 adrenergic receptors & < TLR4 by gene expression	White, et al, 2012
Obstructive sleep apnea	> Plasma sTNFRI	Al-shair, et al, 2011
Hypoxia	Sickness behavior	Sherry, et al, 2009
	>Plasma L-1β & IL-1R1	Basu, et al, 2005
Chronic Obstructive Pulmonary Disease	>Plasma TNFα	Mills, et al, 2008
Breast cancer before treatment	>CMV antibody titers	Fagundes, et al, 2012
Breast cancer soon after treatment	>sTNFRII	Bower et al, 2011b
Breast & prostate cancer with radiation	>Plasma CRP & IL-1Ra	Bower, et al, 2009
Advanced cancer	>Plasma IL-6, TNFα	Kwak et al, 2011
	>Plasma cortisol, ACTH, epinephrine & norepinephrine.	Thornton, et al, 2010
Non-small cell lung cancer with concurrent chemoradiation	>Plasma IL-6, IL-10 & sTNF-R1	Wang, et al, 2010
Testicular cancer, long term survivors	>Plasma IL-1Ra & CRP	Orre, et al, 2009
Breast cancer survivors	>Transcripts for NF-κB & < transcripts for glucocorticoids	Bower et al, 2011a
	>CRP	Orre, et al, 2011
	>HPA and SNS dysfunction	Fernandez-de-las-Penas, 2012
Cancer	>Plasma IL-6, IL-1Rα, neopterin	Schubert, et al, 2007 review

Davis et al., 2008 reported elevation of the pro-inflammatory cytokine, IL-6, in stimulated mononuclear cells from fatigued patients with rheumatoid arthritis (RA). The strongest correlates of fatigue in the RA cohort studied by Stebbings et al. (2010) were depression ($p < 0.001$) and anxiety ($p < 0.001$). Comparing a severely fatigued group of RA patients with cases less fatigued, van Hoogmoed et al. (2010) reported that the proportion of patients negative for rheumatoid factor (RF) was larger in the more fatigued group. However, the DAS-28 (a measure of disease severity), the number of tender joints, swollen joints and the general health rating were significantly worse in the severely fatigued RA patients as were worse pain scores. In contrast, erythrocyte sedimentation rate, hemoglobin level and acute phase reactant C-reactive

protein (CRP) did not differ between the two groups, nor did they correlate with fatigue severity. A marker of inflammation, CRP is produced mainly by hepatocytes, and its production is regulated by IL-6 (Boncler and Watała, 2009). In systemic lupus erythematous (SLE), serum values of CRP were significantly higher ($p < 0.001$) in patients compared with healthy controls. However, CRP did not distinguish disease severity (Rezaieyazdi et al., 2011). According to Lee et al. (2010) patients with SLE often present with flu-like symptoms including fatigue and with high serum interferon (IFN) γ levels.

In 318 individuals with inflammatory bowel disease (IBD), elevated CRP occurred in both Crohn's disease (CD) and ulcerative colitis (UC). However extreme levels of CRP (>20 mg/L) was observed most frequently in CD. High levels of fatigue occurred in 78% of patients with CD and in 67% of those diagnosed with UC (Graff et al., 2011). In another report, CF was found in 29% (14/48) of CD and 22% (20/92) of UC compared to 11% (260/2287) of healthy controls ($p < 0.001$ for both diagnoses). Linear regression analysis confirmed hemoglobin values, present gastrointestinal symptoms, and altered sleep to be the most important predictors of CF (Jelsness-Jørgensen et al., 2011). Fatigue can also be the unintended consequence of therapeutic approaches. A published example is the use of azathioprine or 6-mercaptopurine for treatment of IBD (Lee et al., 2009).

Neurological disorders such as multiple sclerosis (MS) are associated with fatigue. MS patients with comorbid major depressive disorder (MDD) showed normal morning but elevated evening salivary cortisol levels, resulting in a flattened slope. While a higher frequency of tumor necrosis factor (TNF)α and IFNγ production by stimulated CD8+ T cells was also seen in MS patients with MDD, these markers were more closely associated with fatigue than depression (Gold et al., 2011). Cytokine mRNA expression for TNFα was measured by real time polymerase chain reaction (RT PCR) and found to be positively correlated with fatigue in MS cases (Flachenecker et al., 2004). A useful paradigm in biomarker research in fatiguing illnesses involves an exercise challenge model with sampling during and after the challenge.White et al. (2012) used this approach to study patients with MS. Compared to controls, MS cases had greater post-exercise increases than controls in gene expression of β-1 and β-2 adrenergic receptors (1.4 ± 0.27- and 1.3 ± 0.06-fold increases, respectively, $p = 0.02$ and $p < 0.001$) and greater decrease in gene expression for toll-like receptor 4 (TLR4) ($p = 0.02$). Post-exercise, IL-10 and TLR4 decreases correlated with higher fatigue scores.

Hypoxia associated with stroke, heart attacks, lung disease and altitude sickness is often accompanied with sickness behavior, including fatigue, malaise and lethargy (Sherry et al., 2009). Basu et al.,(2005)hypothesized that these symptoms are due to the neuroinflammation subsequent to elevated cytokines and cytokine receptors, particularly elevated IL-1β and IL-1R1. Fatigued subjects with obstructive sleep apnea had elevated plasma soluble TNF receptor 1 (sTNFRI) (Al-shair et al.,2011). Those with chronic obstructive pulmonary disease had elevated plasma TNFα (Mills et al., 2008).

Reported by as many as 40% of cancer patients at diagnosis, cancer-related fatigue (CRF) is a frequent early symptom of malignant disease that often becomes chronic. A review by Schubert et al., (2007) reported significantly positive correlations between fatigue and IL-6 ($r = 0.12$, $p = 0.004$), fatigue and IL-1 receptor antagonist (IL-1 Ra) ($r = 0.24$, $p = 0.0005$), and fatigue and neopterin ($r = 0.22$, $p = 0.0001$). In breast cancer significant fatigue persisted in over 30% of women 10 years following chemotherapy (Bower, 2007). This is a significant patient community with breast cancer accounting for 25% of the $157 billion cost of malignant disease in the US (Radice and Redaelli, 2003). A recent study of breast cancer survivors found increased expression of genes coding for transcripts for NF-κB and decreased expression for glucocorticoids in subjects with fatigue as compared to those without (Bower et al., 2011a). Fernández-de-Las-Peñas et al. (2012) reported that breast cancer survivors with the Met/Met genotype had greater hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) dysfunction that was correlated with severe fatigue. The Met/Met genotype results from substitution of valine with methionine at codon 158 on chromosome 22q11, resulting in lower catechol-O-methyltransferase activity, which may affect estrogen metabolism.

Patients with lung cancer during concurrent chemoradiation therapy developed significant symptom burden including fatigue and elevated plasma IL-6 and sTNFRI (Wang et al., 2010). Liu et al., 2012studied fatigue associated with chemotherapy in breast cancer patients and the inflammatory markers, IL-6, IL-1Ra before and during cycle 1 and cycle 4 of chemotherapy. Fatigue increased, levels of IL-6 increased and IL-1Ra decreased during chemotherapy. Orre et al. (2011) reported that fatigue levels in breast cancer survivors were significantly and positively associated with CRP ($p < 0.001$) and leukocyte count ($p = 0.018$), but not with levels of IL-1Ra, IL-6, sTNF-R1 or neopterin in unadjusted analyses. Only CRP remained significantly associated with fatigue levels in the fully adjusted models ($p = 0.020$). Bower et al. (2011b) examined the relationships of markers of inflammation, IL-1, IL-1Ra, sTNFRII and CRP, to fatigue in women with breast cancer early after primary treatment. Significant correlation was observed only with elevations in sTNFR11. Thornton et al. (2010) looked at co-occurrence of pain, depression, and fatigue in advanced cancer. They reported that these three symptoms were associated with biological mediators including elevated plasma levels of cortisol and adrenocorticotropic hormone indicating HPA activation and elevated plasma epinephrine and norepinephrine indicating sympathetic nervous system activation.

Fagundes et al. (2012) sought to determine biomarkers of fatigue that exist before cancer treatment. Relationships between the expression of latent Epstein–Barr virus (EBV) and cytomegalovirus (CMV) and fatigue were examined in 158 women newly diagnosed with breast cancer or awaiting a positive diagnostic result. Higher CMV antibody titers, but not EBV antibody titers, were associated with a greater likelihood of being fatigued. Associations between fatigue and higher CMV antibody titers remained after controlling for alcohol use, smoking, comorbidities, depressive symptoms, age, BMI, cancer stage, and

sleep problems. More sleep problems and higher levels of depressive symptoms were also associated with a greater likelihood of being fatigued.

Cytokines or their receptors and antagonists were measured in half of the papers on disease-associated fatigue reviewed. However, the cytokines, the methods of analyses and source of samples, plasma, CSF, intracellular in lymphocytes and stimulated cell culture supernatants, varied widely among the studies. Elevated IL-6 was noted in each of the 5 papers were it was measured, as was TNFα. In only 2 of the 5 were results for both proinflammatory cytokines included. The general marker of inflammation, CRP, was elevated in those studies in which it was measured. Dysregulation of the HPA axis and SNS were, as expected, associated with CF. Analytic methods focused on the expression of individual molecules are unlikely to be fruitful in the discovery of clinically useful biomarkers. Incorporation of these markers into integrative network-based analyses will allow identifying associations at the pathway level.

3. Biomarkers of chronic fatigue syndrome/myalgic encephalomyelitis

We agree with the suggestion of Silverman et al. (2010) that CFS/ME is an excellent model for addressing the biology of CF. All cases of CFS/ME have CF, which is required by the case definitions ([Fukuda et al., 1994] and [Carruthers et al., 2011]). Some relevant literature on a related syndrome, gulf war illness (GWI), also associated with CF, is included in this review. Population-based studies estimate the prevalence of CFS/ME at 0.23–0.41% ([Reyes et al., 2003] and [Jason et al., 1999]). Hypothetical initiating events for CFS/ME include infections, stress and exposure to toxins ([Evengård and Klimas, 2002], [Glaser et al., 2005] and [Maes et al., 2007]). The level of immune impairment has convinced several labs involved in the search for new pathogens to look at this illness with new interest. Duration of illness typically exceeds 10 years. Persistence may involve complex interactions of immune, autonomic and neuroendocrine regulation and remains poorly understood ([Bou-Holaigah et al., 1995], [Fuite et al., 2008] and [Hurwitz et al., 2009]). Current CFS/ME treatments are directed at reducing symptom severity but no cure exists for this condition. It follows that this cause of chronic disability has far-reaching consequences and constitutes a significant public health concern and economic burden to society as a whole.

A sample of recent work on molecular diagnosis and associated sub-typing is shown in Table 2. In 22% of the reports cited, cytokines were studied. Fletcher et al. (2009), in a baseline study using a multiplex chemoluminescent method (Quansys, Logan, Utah) of 16 plasma cytokines in 40 CFS/ME patients compared to 59 controls, reported higher levels of IL-4, IL-5, IL-12, lymphotoxin α (LTα), IL-1α, IL-1β, IL-6, lower amounts of IL-8, IL-13, IL-15 and not different IL-2, IFNγ, IL-10, IL-17, IL-23 and TNFα. Brenu et al. (2011) examined supernatants from PHA stimulated peripheral blood cells and found elevated levels of IL-10, IFNγ and TNFα in 95 CFS/ME patients compared to 50 controls, with both groups at rest. Natural killer (NK) cell function (NKCC) was examined by these two research groups. Brenu et al. (2011) andFletcher

et al. (2010a) found NKCC to be low, confirming many past reports. Maher et al. (2005) reported that the number of molecules of intracellular perforin per NK cells, as determined by quantitative intracellular flow cytometry, was related to NK cell function and was low in CFS/ME cases, compared to controls and also in CD3+CD8+ T cells. In contrast, Brenu et al. (2011) measured NK cell perforin by gene expression and reported it elevated in CFS/ME. The discrepancy between these two studies is unlikely to be explained by methodology because both studies reported that perforin levels were low in CD8+ T cells.

Table 2. Biomarkers associated with fatigue in patients with CFS/ME

Biomarkers	References
>IL-10, IFNγ, TNFα by PHA stimulated lymphocytes; >CD4+CD25+ T cells expressing FoxP3 and VPACR2; <cytotoxic activity of NK and CD8+T cells; <granzyme and >perforin by gene expression in CFS/ME compared to HC at rest.	Brenu, et al., 2011
>IL-4, IL-5, IL-12, LTα, IL-1α, IL-1β, IL-6; <IL-8, IL-13, IL-15; ≥IL-2, IFNγ, IL-17, IL-23, TNFα in plasma of CFS/ME compared to HC at rest.	Fletcher, et al., 2009
Cytokine co-expression networks distinct in CFS/ME compared to HC. Subjects at rest.	Broderick, et al., 2010
<Perforin in NK cells and CD8+T cells by quantitative flow cytometry. CFS/ME compared to HC at rest	Maher, et al., 2005
<Perforin by gene expression in GWI compared to HC at VO_2Max in exercise challenge	Whistler, et al, 2009
<NK cell cytotoxicity; < plasma dipeptidyl peptidase IV;>T-cell activation. CFS/ME compared to HC at rest	Fletcher, et al., 2010a
In CFS/ME compared to HC: absence of significant increase in IL-6 & TNFα following exercise challenge	Jammes, et al., 2009
IL-1β, IL-12, IL-6, IL-8, IL-10, and IL-13 elevated at 8 hrs post exercise in subjects showing symptom flair at 48 hrs.	White, et al, 2010
>NPY in CFS/ME subjects compared to HC at rest;	Fletcher, et al., 2010b
no exercise related change for NPY, IL-6, IL-10, IL12, TNFα in CFS/ME but > in HC	Harvey, et al., 2011
<Serum vitamin E, a marker for oxidative stress in CFS/ME compared to HC at rest	Miwa & Fujita, 2010
Exercise related <plasma F(2)-isoprostanes (marker of oxidative stress); No effect of exercise on plasma IL-6 or sIL-6R in CFS/ME or HC.	Robinson, et al., 2010
In most CFS/ME but not in HC, exercise >transcription for most sensory and adrenergic receptors and one cytokine which correlated with fatigue and pain.	Light, et al, 2012
Metabolic syndrome predictors elevated in CFS/ME compared to HC at rest.	Maloney, et al, 2010
Increased lactate levels in ventricular cerebrospinal fluid of CFS/ME compared to HC at rest.	Murrough et al. 2010
Significant deficiencies in mitochondrial function in CFS/ME compared to HC at rest.	Myhill et al., 2009
Quantitative proteomics using high resolution mass spectrometry of CSF, unique patterns associated with CFS compared to HC and Lyme disease at rest.	Schutzer, et al., 2011
Unique CFS/ME spinal fluid proteome of 60 proteins when compared to HC and GWI. The CFS/ME and GWI patients shared 20 unique proteins at rest.	Baraniuk et al 2005
> CRP, >8-iso-prostaglandin F(2 alpha) isoprostanes in CFS/ME compared to HC at rest	Spence, et al, 2008
<LPS induced pro-inflammatory cytokines under psychological stress in CFS/ME compared to HC at rest	Gaab et al, 2005
> CRP in CF cases not meeting the CFS/ME definition; no difference between CFS/ME and HC at rest	Raison et al, 2009
Abnormal pattern of cortisol over 24 hours associated with elevated fatigue.	Torres-Harding, et al 200
<Cortisol levels and flattened diurnal release of cortisol) associated with a poorer response to CBT in CFS/ME.	Roberts, et al., 2010
Variations in the 5' region of NR3C1 (glucocorticoid receptor gene) in CFS/ME compared to HC at rest.	Rajeevan et al., 2007
HPA axis dysfunction in CFS/ME compared to HC at rest.	Papadopoulos & Cleare,
HPA axis dysfunction CFS/ME compared to HC at rest.	Ben-Zvi, et al., 2009
No evidence for biomarkers using gene expression in a twin study.	Byrnes et al., 2009
Significant evidence for a heritable contribution to predisposition to CFS/ME.	Albright, et al, 2011
Gene expression revealed 'CFS signature genes'.	Kerr, et al., 2008
Reassessment of 'CFS signature genes' failed to confirm predictive ability.	Frampton, et al., 2011

Full-size table

White et al. (2010) measured cytokines using a multiplex bead-based system (Luminex Corp, Austin, TX) in serum from 19 CFS/ME and 17 controls collected at several time points pre/post a moderate exercise challenge. In contrast to the results of Fletcher et al., no differences were found at baseline. However, 48 h after the challenge, 11 patients showed symptom flair. In that group, IL-1β, IL-12, IL-6, IL-8, IL-10,

and IL-13 were elevated at 8 h post exercise. Jammes et al. (2009) and Robinson et al. (2010) also reported a differential effect of aerobic exercise on plasma cytokines. Both studies found that the exercise elevated plasma IL-6 in controls but had no effect in CFS/ME. As shown in Table 3, an aerobic exercise challenge increased differences between CFS/ME cases and healthy controls on 8 of 9 biomarkers, plasma NPY, IL-5, IL-6, IL-10, IL-12 and TNF. Samples were drawn at baseline, $V0_2$ max and 4 h. It should be noted that methodology and sensitivity for cytokine measurement varied in these studies.

Table 3. Effect of aerobic exercise challenge on biomarkers in CFS/ME

	Friedman Test Result: Significant Values ($p < .05$) in Bold	
	CFS/ME (N=23)	HC (N=34)
%CD26+CD2+ (T & NK cells)	**<.000 (↓)**	**<.000 (↓)**
rMolCD26/CD2+ (T & NK cells)	**0.004 (↓)**	**<.000 (↑)**
NPY (pMol/L plasma)	0.436	**<.000 (↑)**
IL-6 (pg/ml plasma)	0.607	**0.008 (↑)**
IL-10 (pg/ml plasma)	0.857	**0.001 (↑)**
IL-12p70 (pg/ml plasma)	0.354	**0.002 (↑)**
TNFα (pg/ml plasma)	0.624	**0.007 (↑)**
rMolPerforin/NK cell	**0.012 (↑)**	**<.000 (↑)**
NKCC (%)	**0.023 (↑)**	**0.001 (↑)**

Gulf War Illness (GWI) patients demonstrated impaired immune function as demonstrated by decreased NKCC and altered gene expression associated with NK cell function, including perforin. Pro-inflammatory cytokines, T-cell ratios, and salivary cortisol) were also altered in GWI cases compared to control subjects. A three point exercise challenge augmented these differences, with the most significant effects observed immediately after $V0_2$Max, possibly implicating some block in the NK and CD8 T-cells ability to respond to “stress-mediated activation”. This result has positive implications for the development of laboratory diagnostic tests for GWI and provides a paradigm for exploration of the immuno-physiological mechanisms that are operating in GWI, and similar complex syndromes (Whistler et al., 2009).

Light et al. (2009) demonstrated, with real-time, quantitative PCR, that 19 CFS patients had lower expression of beta-2 adrenergic receptors but otherwise did not differ from 16 control subjects before exercise. After a sustained moderate exercise test, CFS patients showed greater increases than control subjects in mRNA for genes that can detect increases in muscle produced metabolites (ASIC3, P2X4, P2X5), genes that are essential for SNS processes (adrenergic α-2A, β-1, and β-2, as well as COMT), and immune function genes (IL10, and TLR4) lasting from 0.5 to 48 h ($p < 0.05$). In a more recent study of moderate exercise effects on gene expression, Light et al. (2012) used a 25 min exercise challenge with blood sampling at 0.5, 8, 24 and 48 h post exercise. The relative mRNA values from the four time-points were summed into a single measure labeled area under the curve (AUC) and then log transformed. No gene expression changes occurred following exercise in controls. In 71% of patients with CFS/ME, moderate exercise increased most sensory and adrenergic receptor’s and one cytokine gene’s transcription for 48 h. These post-exercise increases correlated with behavioral measures of fatigue and

pain. In contrast, for the other 29% of patients with CFS/ME, adrenergic α-2A receptor's transcription was decreased at all time-points after exercise; other genes were not altered. A history of orthostatic intolerance was significantly more common in the α-2A decrease subgroup.

Spence et al. (2008) reported increased plasma concentrations of CRP in patients with CFS/ME. In their study of 41 patients with CFS/ME and in 30 healthy subjects, plasma CRP in CFS/ME was 2.58 ± 2.91 compared with 1.07 ± 2.16 mg/L in HC; $p < 0.01$). They also found elevation of a marker for oxidative stress, 8-iso-prostaglandin F(2 alpha) isoprostanes (470.7 ± 250.9 compared with 331.1 ± 97.6 pg/ml respectively; $p < 0.005$). In a population-based sample in metropolitan, urban and rural areas of Georgia, CDC researchers screened 10,837 households with 21,165 residents (Raison et al., 2009). When examined as a categorical variable (based on a cut-off of >3 mg/L), CRP was significantly higher in subjects with CFS/ME (34.38%) and in fatigued individuals who did not fulfill the diagnostic case definition (ISF) (38.05%) than in healthy controls (20.72%) (CFS/ME: OR = 2.00, 95% CI = 1.08–3.74; ISF: OR = 2.35, 95% CI = 1.38–4.00). Other variables associated with CRP >3 mg/L included sex, race, PCS score, BMI, and SDS depression score. After adjustment for age, sex, race, location of residence, BMI, depressive status and use of immune modulating medications, subjects classified as ISF continued to demonstrate increased CRP (adjusted OR = 2.34, 95% CI = 1.29–4.27, $p = 0.0120$). After adjustment, the association between CRP >3 mg/L and CFS/ME did not remain significant (adjusted OR = 1.62, 95% CI = 0.75–3.53, $p = 0.8569$). In the Copenhagen General Population Study of approximately 63,500 individuals, the distribution of circulating levels of CRP was markedly skewed to the right with 97% of the participants having CRP levels of <10 mg/L. The median plasma CRP concentration was 1.53 mg/L (IQR, 1.14–2.51) and 34% of the participants had circulating CRP levels of ☐2 mg/L (Allin and Nordestgaard, 2011).

Papadopoulos and Cleare (2011) concluded in a recent review that the clinically relevant HPA axis dysfunction occurring in CFS/ME patients includes mild hypocortisolism with attenuated diurnal variation of cortisol, enhanced negative feedback to the HPA axis and blunted HPA axis responsiveness. Dysregulation of the HPA axis was associated with a number of neuroimmune disorders including CFS/ME, which was characterized by a hypoactive rather than a hyperactive HPA axis (Roberts et al., 2010). Lattie et al. (2012) reported that CFS/ME cases with greater perceived stress management skills had less fatigue ($p = 0.019$) and emotional distress ($p < 0.001$), greater diurnal cortisol slope ($p = 0.023$) and lower IL-2 levels ($p = 0.043$). The influence of stress management skills on distress and fatigue appeared greatest among patients who had elevated IL-6 levels. Gaab et al., 2005 used the Trier Social Stress Test (TSST) to determine that while cortisol responses to stress were normal, LPS induced pro-inflammatory cytokine levels were significantly attenuated in CFS/MS patients. CFS/MS patients showed an inverse response pattern in comparison to healthy controls, i.e. stimulated cytokine production decreased shortly after stress in CFS/ME patients, while it increased in controls. Torres-Harding et al.

(2009) reported on the relationships between salivary cortisol levels and illness symptomatology in a group of 108 individuals with CFS/ME. Results indicated that fatigue and pain were associated with salivary cortisol levels. In particular, variance from the expected pattern of cortisol over 24 h was associated with increased levels of fatigue. Glucocorticoid receptor gene NR3C1 was implicated as a potential mediator of CFS/ME, and suggested variations in the 5′ region of NR3C1 as a possible mechanism through which the alterations in HPA axis regulation and behavioral characteristics of CFS/ME may manifest (Rajeevan et al., 2007).

A marker for chronic immune activation, dipeptyl peptidase IV/ CD26 was shown to be dysregulated in CFS/ME (Fletcher et al., 2010a). A substrate for this dipeptidase is the stress hormone, neuropeptide Y (NPY), which was elevated in CFS/ME cases (Fletcher et al., 2010b) and correlated with symptom severity including fatigue.

Schutzer et al. (2011) used liquid chromatography coupled to high-resolution mass spectrometry based label-free quantitative proteomics approach to examine cerebral spinal fluid (CSF) samples from patients with CFS/ME, Lyme disease and healthy controls. Both patient groups could be distinguished from each other and from controls based on their unique CSF proteins ($p < 0.01$). CFS/ME ($n = 43$) had 2,783 non-redundant proteins, Lyme disease ($n = 25$) 2,768 proteins, and healthy normal controls, 2,630 proteins. Four components (C1S, C4B, C1QB, C1QC) that are seen with activation of the complement cascade were differentially increased in abundance consistently across the Lyme patients compared to CFS/ME. An earlier pilot study published by James Baraniuk's group (2005) compared pooled spinal fluids from 10 CFS/ME patients, 10 GWI patients and 10 controls. They identified a unique CFS/ME spinal fluid proteome of 60 proteins when compared to healthy controls and GWI, though the CFS/ME and GWI patients shared 20 unique proteins (Baraniuk et al. 2005). Among persons with CFS/ME, the number of metabolic syndrome factors was significantly correlated with worse fatigue on a standardized summary measure of fatigue ($r = 0.20$, $p = 0.04$) (Maloney et al., 2010). As proteins contribute directly and indirectly to the regulation of metabolite levels it is not surprising that recent neuroimaging reported by Murrough et al. (2010) has shown increased lactate levels in ventricular cerebrospinal fluid of CFS/ME patients. Similarly, using a targeted assay of adenosine triphosphate (ATP) availability in circulating blood neurophils, Myhill et al. (2009) pointed to significant deficiencies in mitochondrial function in CFS/ME. Though narrowly focused these studies emphasize the importance of metabolite profiling as a direct means of assessing fundamental inefficiencies in cell metabolic function. Serum vitamin E (alpha-tocopherol) concentrations were significantly lower in CFS patients as compared with the control subjects, suggesting increased oxidative stress in the former (Miwa and Fujita, 2010).

Interacting with and orchestrating cell signal transduction and metabolism is the gene regulatory machinery. Broderick et al. (2006) analyzed the correlation patterns in 117 clinical variables measured in 111 female subjects and used these to isolate gene co-expression patterns characteristic of CFS/ME.

These described associations between 17 transcripts related to basic cellular processes involved in cell signaling, ion transport and immune system function. The single most influential of these was sestrin 1 (SESN1), supporting the involvement of oxidative stress in CFS/ME. Kerr et al. (2008) reported on 'CFS signature genes' with predictive power. This set had only five genes out of 44 related to immune regulation. Recently, they have reassessed the CFS/ME disease predictive genes in the original study data and assessed the ability of the proposed 44-gene classifier set to discriminate between CFS/ME patients and healthy control individuals. This classifier was able to discriminate correctly between CFS/ME and healthy control samples in 95% of the training samples. However, when assessed on a new, blinded 128-sample test set only 58% of samples were predicted correctly. Using a variety of methods, it was demonstrated that the 44-gene classifier set did not robustly identify patients with CFS/ME (Frampton et al., 2011). Byrnes et al. (2009) were unable to identify a candidate biomarker for CFS/ME when comparing identical twin sets discordant for illness.

Utah researchers with access to computerized genealogical resource linking multiple generations of genealogy data with medical diagnosis data reported significant evidence for a heritable contribution to predisposition to CFS/ME. Significant excess relatedness was observed for both close ($p < 0.001$) and distant relationships ($p = 0.010$). Significant excess relative risk for CFS/ME was found between first (2.70, 95% CI: 1.56–4.66), second (2.34, 95% CI: 1.31–4.19), and third degree relatives (1.93, 95% CI: 1.21–3.07) (Albright et al., 2011).

Post-exercise mRNA increases for metabolite-detecting receptors were unique to patients with CFS/ME, whereas both patients with MS and patients with CFS showed abnormal increases in adrenergic receptors. Among patients with MS, greater fatigue was correlated with blunted immune marker expression (White et al., 2010). These post-exercise increases correlated with behavioral measures of fatigue and pain.

In current studies by the Miami/Alberta group, GWI and CFS/ME patients showed distinct differences from each other and HC that became dramatically apparent under physiological challenge. While no genes were expressed at rest with a 2-fold difference (false discovery rate FDR < 0.05) in CFS/ME over control subjects we found 18 such genes differentially expressed at rest in GWI. However, CFS/ME subjects were easily distinguished from healthy control subjects under effort due to a dramatic unresponsiveness to exercise. Indeed in moving to peak effort from rest 166 genes became differentially expressed from rest in healthy controls. In contrast 50 genes responded to challenge at peak effort in GWI but only one was expressed in CFS/ME at peak effort versus rest. This lack of early response in the transition from rest to peak effort explains in large part why 466 genes were expressed with a 2-fold difference (FDR < 0.05) in CFS/ME versus control subjects at peak effort as opposed to 28 genes in GWI. Therefore while GWI showed a partial early response to maximal exercise challenge CFS/ME subjects were largely unresponsive in that time frame. Results such as these emphasize how the use of an exercise challenge

to probe the dynamics of response offers a more sensitive measure of the differences separating these patient populations (Broderick et al., 2011). Using a methodology originally developed by Efron et al. (2007), Broderick et al. went onto show that these differences in gene expression implicated 90 documented pathways with the majority being linked to immune metabolism. Examining these pathways as part of an integrated biological system the latter demonstrated that significant differences exist between CFS, GWI and healthy control subjects in terms of the architecture of their active pathway networks. Indeed these significant differences in the recruitment, shedding and re-assignment of regulatory interactions would not be seen using conventional analytic methods focused on the expression of individual markers.

4. Conclusions

Fatigue research faces a number of methodological issues that continue to challenge the biological relevance and clinical impact of the search for useful biomarkers. In particular is the need to describe and understand the structure of complex biological networks. This will require applying novel measures to identify network functional modules within and across levels of biology, and identifying associations at the pathway level to promote increased biological relevance. Because the body operates as an autonomous, fully integrated and self-regulating system it is should not be surprising that even localized muscle fatigue will present systemic biomarkers. One can view the observed myriad of individual biomarkers as being partially overlapping manifestations of a much more fundamental and unified set of biological processes. Closely associated with the single biomarker paradigm is the conventional model of dysfunction originating from the outright failure of a single physiological component. A highly complementary paradigm and one that embraces the overarching regulatory architecture of human physiology is the concept of loss of regulatory performance. This is akin to a controller becoming de-tuned over time and under sustained adaptive load. This manifests as a change in the dynamics of response. An appropriate example would be observations of a fight-or-flight axis response to a stressor that is delayed, develops more slowly and is blunted in amplitude. The recent paper by Broderick et al. (2010) approached the question of cytokine involvement in CFS/ME pathogenesis. Multiplex cytokine data (Fletcher et al., 2009) was used to construct the cytokine co-expression networks, which clearly distinguished cases from controls reinforcing the importance of immune context and relative expression (Fig. 1).

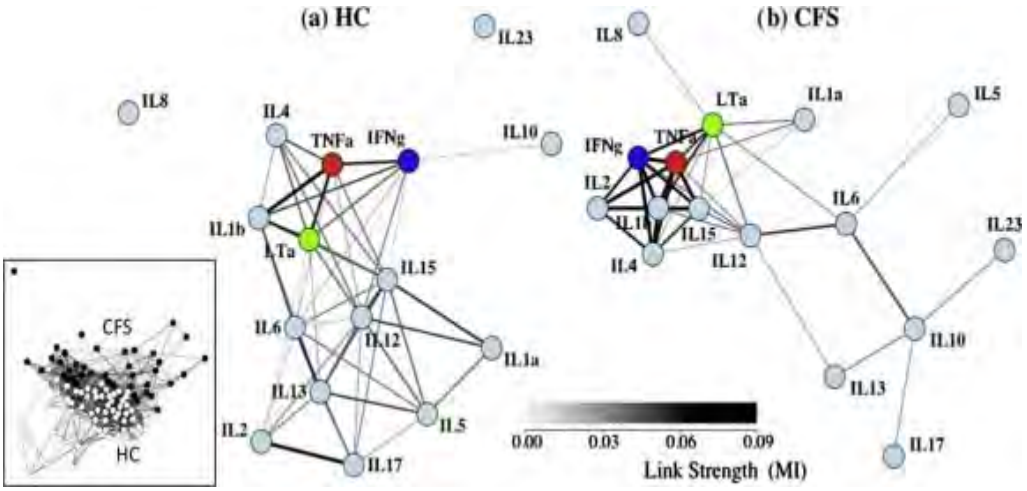

Fig. 1. Networks of cytokine association show visibly different topologies. A weighted spring-electrical embedding structurally reveals the subject–subject (inset) and cytokine–cytokine associations based on measurements in 59 healthy control subjects (a) and 40 CFS/ME patients (b). All edge weights are significant at *p* ☐ 0.01. Separation of subjects was consistent with their assignment to diagnostic groups supporting the use of within-group variation in the estimation of mutual information for cytokine–cytokine associations.

We propose that persistent disorders such as CFS/ME and CRF likely correspond to alternative homeostatic states enabled by the body as an adaptive strategy. These alternate modes of homeostatic control result in a loss of function that is persistent because these configurations are energetically stable (Fig. 2). At and around these new homeostatic states we expect a characteristic change in how the body maintains homeostasis and that this new regulatory program will support different patterns of biomarker association. For example the immune system in these individuals might now use an alternate signaling network, one that may be much less efficient. Empirically mapping networks of endocrine and immune signaling in CFS/ME and CRF will allow us to identify key changes in their basic architecture and in their capacity to support the flow of regulatory information. This will not only enhance the characterization of these conditions but also provide new insight into the regulatory processes that support fatigue as a protective element. Physiological integration and the closed loop regulation spanning broadly across the immune, autonomic, endocrine systems as well as at the level of intracellular signaling and metabolic function may force a paradigm shift in our approach to complex illness as illustrated in Fig. 2. Indeed this type of integrative network-based analysis may be required to progress to the next chapter in our understanding of the biology of fatigue.

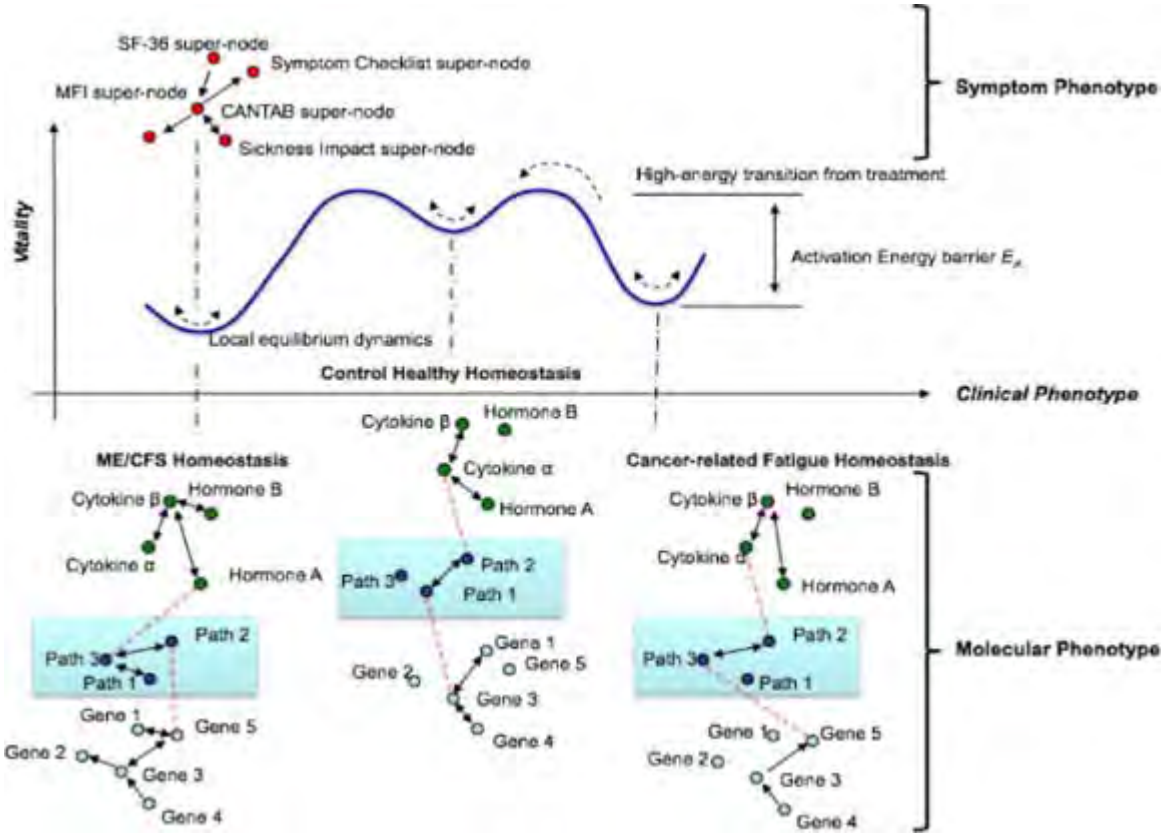

Fig. 2. Diagrammatic representation of the hypothesis whereby two complex and very distinct illnesses CFS/ME and cancer-related fatigue occupy distinct energy-minimal equilibrium points characterized by alternative configurations of the neuroendocrine-immune biomarker and symptom association networks.

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Title: Altered Immune Pathway Activity under Exercise Challenge in Gulf War Syndrome

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Abstract: Though potentially linked to the basic physiology of stress response we still have no clear understanding of Gulf War Syndrome (GWS), a debilitating illness presenting with a complex constellation of immune, endocrine and neurological symptoms. Here we compared male GWS (n=20) with healthy veterans (n=22) and subjects with chronic fatigue syndrome/ myalgic encephalomyelitis (CFS/ME) (n=7). Subjects were assessed using a Graded eXercise Test (GXT) with blood drawn prior to exercise, at peak effort (VO2 max) and 4-hours post exercise. Gene expression in peripheral blood mononuclear cells (PBMCs) was measured using the Affymetrix HG U133 plus 2.0 microarray. The activity in documented pathways was computed for individual samples and compared across groups and exercise phases using a 2-way ANOVA corrected for multiple comparisons (q statistic). GWS was characterized ($q<0.05$) by increases in neuroendocrine-immune signaling and inflammatory activity cast against decreases in apoptotic signaling. Conversely, cell cycle progression and immune signaling were broadly subdued in CFS. Partial correlation was used to construct association networks linking pathways and their interactions with symptom severity. In 27 symptom and demographic variables, 17 correlated directly with the activity of at least one pathway in one of the 3 exercise phases. Interactions with NF-κB and associated pathways that are consistent with altered cholinergic signaling were the dominant theme underlying symptom expression. Findings supported further evaluation of animal models involving stress-potentiated response to pyridostigmine bromide (PB) exposure.

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Strong convictions regarding a specific aetiology for Gulf War Syndrome

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Attn: Dr. Keith W. Kelley, Editor-in-Chief, Brain, Behavior, and Immunity

Subject: Submission of manuscript

Dear Dr. Kelley,

We wish by the following to submit for review by the editorial team of *Brain, Behavior, and Immunity* the manuscript entitled:

"Altered Immune Pathway Activity under Exercise Challenge in Gulf War Syndrome"

In this paper we continue our investigation of immune dysfunction in Gulf War Syndrome (GWS) by surveying changes in pathway activity at three stages of a maximum exercise challenge. To achieve this we used a novel approach whereby we project microarray gene expression profiles from circulating lymphocytes onto the known regulatory circuitry of pathways documented in the National Cancer Institute/ Nature pathway interaction database (PID). Our analysis suggests that lymphocyte pathway activation in Gulf War syndrome (GWS) is significantly different from that found in a sister illness; chronic fatigue syndrome (CFS/ME). GWS presented with decreased apoptotic and TNF-mediated signaling accompanied by increased neurotransmission and inflammatory pathway activity. Conversely, cell cycle progression and immune signaling pathways were broadly subdued in CFS while ephrin A (Eph-A) signaling was increased. Importantly, we constructed interaction networks linking the activation of pathways with changes in 27 symptom and demographic variables in GWS. These showed that symptom severity in GWS was closely associated with the activity of master transcriptional regulator NF-κB. Based on these results we propose that GWS and CFS/ME may involve illness-specific alterations to Wnt/ Ca2+/ NFAT/ NF-κB signaling and the intersecting PI3K, Akt/PKB, Eph (mTOR) axes.

We believe that these results will be of direct relevance and of broad interest to the readership of the journal. Moreover, it is our hope that this work will emphasize the benefits of a systems-based approach in the study of complex disease.

Kind regards,

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Associate Professor, Department of Medicine

Research Highlight

Gulf War syndrome (GWS) may involve illness-specific alterations to Wnt/ Ca2+/ NFAT/ NF-κB signaling and intersecting PI3K, Akt/PKB, Eph (mTOR) axis.

Altered Immune Pathway Activity under Exercise Challenge in Gulf War Syndrome

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Abstract

Though potentially linked to the basic physiology of stress response we still have no clear understanding of Gulf War Syndrome (GWS), a debilitating illness presenting with a complex constellation of immune, endocrine and neurological symptoms. Here we compared male GWS (n=20) with healthy veterans (n=22) and subjects with chronic fatigue syndrome/ myalgic encephalomyelitis (CFS/ME) (n=7). Subjects were assessed using a Graded eXercise Test (GXT) with blood drawn prior to exercise, at peak effort (VO2 max) and 4-hours post exercise. Gene expression in peripheral blood mononuclear cells (PBMCs) was measured using the Affymetrix HG U133 plus 2.0 microarray. The activity in documented pathways was computed for individual samples and compared across groups and exercise phases using a 2-way ANOVA corrected for multiple comparisons (q statistic). GWS was characterized ($q<0.05$) by increases in neuroendocrine-immune signaling and inflammatory activity cast against decreases in apoptotic signaling. Conversely, cell cycle progression and immune signaling were broadly subdued in CFS. Partial correlation was used to construct association networks linking pathways and their interactions with symptom severity. In 27 symptom and demographic variables, 17 correlated directly with the activity of at least one pathway in one of the 3 exercise phases. Interactions with NF-κB and associated pathways that are consistent with altered cholinergic signaling were the dominant theme underlying symptom expression. Findings supported further evaluation of animal models involving stress-potentiated response to pyridostigmine bromide (PB) exposure.

Keywords: gene expression, pathway activity, Gulf War Illness; exercise challenge; mathematical immunology.

1. Introduction.

Within months of returning from Operation Desert Storm an alarming number of Gulf War veterans began reporting fatigue, musculoskeletal discomfort, skin rashes, and cognitive dysfunction (Haley, 1997; Fukuda et al., 1998; Wolfe et al., 1998). We still have no clear understanding of Gulf War Syndrome (GWS) although the basic physiology of response to stress whether psychological, chemical or other provides a starting point. Indeed clinical presentation of GWS overlaps strongly with that of another stress-mediated illness: Chronic Fatigue Syndrome/ myalgic encephalomyelitis (CFS/ME) (Kang et al., 2003; Eisen et al., 2005). Though brain imaging has shown promise (Liu et al., 2011; Haley et al., 2009), single biomarkers for these illnesses in the lymphocyte transcriptome remain elusive (Byrnes et al., 2009). Early RT-PCR amplification of cytokine associated transcripts by Zhang et al., (1999) indicated that Gulf War veterans diagnosed with chronic fatigue syndrome (CFS/ME) had significantly higher levels of IL-2, IL-10, IFN-g, and TNF-a transcript than healthy controls while non-veteran CFS/ME subjects did not differ. More recent work by our group (Whister et al., 2009) focused on the expression in Gulf War veterans of transcripts associated with natural killer (NK) cell function before, during and after a maximal graded exercise challenge (VO2 max.). In this specific group of 108 microarray probe sets, 49 were increased in expression 2-fold and none were decreased in GWS. Specifically, mRNA for perforin (PRF1) and granzyme (GZMB), along with killer cell lectin-like receptors (KLR) and death receptor induction (FASLG) indicated a depressed NK cell cytotoxic response to exercise in GWS.

Additional surveys have been done using animal models and have focused on exposure to chemical agents used in theatre; namely organophosphates and acetylcholine esterase (AChE) inhibitors such as pyridostigmine bromide (PB) (Golomb

2008, Amourette et al., 2009) and sarin (Shewale et al., 2012; Mach et al., 2008). Though much of this has focused on proteomic and phospho-proteomic profiling (Torres-Altoro et al., 2011; Zhu et al., 2010), recent work by Barbier et al., (2009) surveyed the expression of genes associated with stress response, learning and memory in the hippocampus and hypothalamus of mice. Their results indicated that stressed animals exposed to PB showed increases in hippocampal expression of three genes implicated in memory development: brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase B (TrkB) and calcium /calmodulin-protein kinase II alpha (CamKII). In studies such as these the focus has been on a specific selection of genes rather than on genome-wide profiling. As a result the analysis has relied on more traditional statistical methods that consist in comparing the expression levels of one gene at a time.

Because of the extensive connectivity found in regulatory networks at all levels of biology, our group has attempted to cast individual molecular messages in the context of broad-scale immune signaling and cellular demographics (Broderick et al., 2011). Here, we extended this type of integrative approach to the analysis of gene expression by incorporating a priori knowledge of biological pathways. Specifically, we applied a novel methodology (Efroni et al., 2007; 2008) for identifying gene expression status and the activity level of the pathways they support as described in the National Cancer Institute (NCI)/ Nature Pathway Interaction Database (PID) (Schaefer et al., 2009) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2010). Illness groups and the subjects that compose them were then compared in terms of the activity level of these pathways and constituent pathway segments. This is a significant departure from conventional analysis which is based on only the subset of pathways supported by genes that change significantly in expression across groups (Kerr et al. 2008a; Kerr et al. 2008b; Whistler et al. 2009). In the more conventional approach no

estimate of the level of pathway activation is produced and because gene expression values must be averaged across groups, detail at the level of individual subjects is lost. Using the alternative approach for pathway analysis described above we compared the gene expression in peripheral blood mononuclear cells (PBMC) collected before exercise, at peak effort and 4 hours after exercise in Gulf War veterans suffering from GWS with that of healthy veterans. We also use a smaller pilot cohort of subjects diagnosed with CFS/ME as a disease control group. Using a 2-way ANOVA and correcting for false discovery we found 20 pathways uniquely expressed in GWS compared to CFS/ME subjects and healthy subjects. These described increased neuro-immune and endocrine-immune signaling and decreased control of cell cycle and apoptosis. Association networks based on partial correlation were constructed linking key measures of symptom severity with pathway activation. These indicated a broad support of symptom presentation by sub-networks of pathways involved in cholinergic immune signaling. These findings reinforced the potential value of animal models that capture the stress-induced potentiation of physiological response to PB exposure.

2. Material and Methods.

2.1 Sample collection and processing.

2.1.1 Cohort recruitment. As part of a larger ongoing study a subset of CFS/ME (n=7), GWS subjects (n=20) and healthy but sedentary Gulf War era veterans (n=22) were recruited from the Miami Veterans Administration Medical Center. All subjects were comparable in age, body mass index (BMI), ethnicity and duration of illness. Subjects were male and ranged in age between 30 and 55. Inclusion criteria was derived from Fukuda et al. (1998), and consisted in identifying veterans deployed to the theater of operations between August 8, 1990 and July 31, 1991, with one or more symptoms

present after 6 months from at least 2 of the following: fatigue; mood and cognitive complaints; and musculoskeletal complaints. Subjects were in good health prior to 1990, and had no current exclusionary diagnoses (Reeves et al., 2003). Medications that could have impacted immune function were excluded. Use of the Fukuda definition in GWS is supported by Collins et al. (2002). Summary results of subject demographics and exercise performance are listed in Table 1.

Ethics statement. All subjects signed an informed consent approved by the Institutional Review Board of the University of Miami. Ethics review and approval for data analysis was also obtained by the IRB of the University of Alberta.

2.1.2 Subject assessment. All subjects received a physical examination and medical history including the GWS symptom checklist as per the case definition. Pysychometric questionnaires included the Multidimensional Fatigue Inventory (MFI) (Smets et al., 1995), a 20-item self-report instrument designed to measure fatigue, and the Medical Outcomes Study 36-item short-form survey (SF-36) (Ware and Sherbourne, 1992) assessing health-related quality of life. The Krupp Fatigue Severity Inventory (Krupp FSI) was also used to measure perceptions of fatigue severity (Krupp et al., 1989) while the impact of symptoms on the activities of daily life was measured with the Sickness Impact Profile (SIP) (Bergner et al., 1981). Subjects were screened for quantity and quality of sleep, and evaluated for the likelihood of primary sleep disorders using the Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989). Designed to assess symptoms of post-traumatic stress disorder (PTSD), the Davidson Trauma Scale (DTS)(Davidson et al., 1997) was applied to those subjects who reported a traumatic experience (death of loved one, assault, injury, etc…). This instrument is divided into three components: intrusion, avoidance, and hyper-arousal. While sixty-six percent of the GWS cohort presented DTS

scores consistent with PTSD, this data is available only for a small fraction of CFS/ME subjects and healthy control subjects. Finally, aspects of cognitive impairment were assessed using the Paced Auditory Serial Addition Task (PASAT). This serial-addition task is used to assess the rate of information processing, sustained attention, and working memory (Gronwall et al., 1977). A summary of symptom severity measures and results obtained in each group is available in supplemental table S1.

Immune response was stimulated with a standard Graded eXercise Test (GXT) using a Vmax Spectra 29c Cardiopulmonary Exercise Testing Instrument, Sensor-Medics Ergoline 800 fully automated cycle ergometer, and SensorMedics Marquette MAX 1 Sress ECG. According to the McArdle protocol (McArdle et al., 2007) subjects pedaled at an initial output of 60 watts for 2 minutes, followed by an increase of 30 watts every 2 minutes until the subject reached: 1) a plateau in maximal oxygen consumption (VO2); 2) a respiratory exchange ratio >1.15; or 3) the subject stopped the test. A first blood draw was conducted prior to exercise following a 30-minute rest. Second and third blood draws were conducted upon reaching peak effort (VO2 max) and at 4-hours post exercise respectively.

2.1.3 Gene expression. At each blood draw three 8-mL tubes of blood were collected in CPT vacutainers (B-D- Biosciences, San Jose, CA). The peripheral blood mononuclear cells (PBMC) were isolated and stored in liquid nitrogen under conditions designed to maintain viability. Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. The quality and quantity of RNA was assessed using the Agilent Bioanalyzer 2100 RNA 6000 Nano Kit (Agilent Technologies, CA). From each sample, 300 ng of total RNA was converted into cDNA by reverse transcription using a T7-oligo(dT) primer and the Affymetrix 3' IVT Express Kit

(Affymetrix, Santa Clara, CA) according to standard manufacturer protocol. The generated cDNA was purified using the GeneChip Sample Cleaning Module (Affymetrix) and labeled cRNA was generated by in vitro transcription using the biotinylated nucleotide mix. This was then purified with the Cleaning Module and quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE USA). In each preparation 11µg cRNA was fragmented in Fragmentation Buffer (Affymetrix) in a final reaction volume of 25 µl.

Hybridization, washing, staining and scanning were done using Affymetrix GeneChip instruments (Hybridization Oven 640, Fluidics Station 450Dx, Scanner GCS3000Dx) and Affymetrix Human U133 2.0 arrays (Affymetrix) as per manufacturer's standards. Microarray image files (.cel data) were generated using the Affymetrix GCOS software tool with default microarray analysis parameters to provide overall within chip normalization of the image intensity distribution. The quality parameters that were monitored besides cRNA total yield and cRNA A260/A280 ratio included: (i) background noise (Q value), (ii) percentage of present called probe sets, (iii) scaling factor, (iv) information about exogenous Bacillus subtilis control transcripts from the Affymetrix Poly-A control kit (lys, phe, thr, and dap), and (v) the ratio of intensities of 3' probes to 5' probes for a housekeeping gene (GAPD).

2.2 Numerical analysis

2.2.1 Comparative analysis of gene expression. Prior to analysis raw microarray data was corrected for chip-to-chip variability. Removal of background signal intensity was performed using GeneChip RMA (GC-RMA) (Wu et al., 2004; Wu and Irizarry, 2005; Katz et al., 2006), an extension of classical Robust Multichip Average (RMA) algorithm (Irizarry et al. 2003). The range of intensity values were adjusted using quantile

normalization (Bolstad et al. 2003). Expression values were transformed logarithmically with base 2 (log2) to improve normality. The significance of changes in gene expression across subject groups and across all 3 time points were evaluated using a 2-way analysis of variance (ANOVA-2). In all cases, null probability values were adjusted for multiple comparisons using the method proposed by Storey (2002) for estimating the false discovery rate (FDR) and probability of false discovery *q*. Gene and pathway associations of Affymetrix probe sets were obtained using the Protein Analysis THrough Evolutionary Relationships (PANTHER) software system (Mi et al., 2010).

2.2.2 Estimating Pathway Activity. Individual gene products are expressed in a highly coordinated manner that supports the function of biochemical reaction pathways in the cell. In accordance with this we have used a novel algorithm developed by Efroni et al. (2007; 2008) to estimate the activity of known pathway segments from the expression of the genes that encode their components. The first step consists of converting a continuous measure of gene expression into a discrete gene status, namely up-expressed or down-expressed. In brief, for every individual gene the distribution of expression values across all samples are numerically fit to two separate gamma probability distribution functions: one describing the distribution of expression values supporting the up state and one describing the distribution of values supporting the down state. The expectation maximization (EM) algorithm is applied to provide a set of maximum-likelihood estimates for the parameters a_i and b_i as well as the values of the mixture coefficients η_i that weigh the contribution of the gamma distribution (Eq. 1-3) for each state i=1,2. The vector of these parameters is estimated iteratively from Θ^0 to Θ such that the function $Q(\Theta,\Theta^0)$ is maximized (Eq. 1).

$$Q(\theta,\theta^0)=\sum_t\sum_i \omega_{t,i}(\log(\eta_i)-\log(\gamma(x_t;a_i,b_i)) \quad \textbf{(1)}$$

Where

$$\gamma(x_t;a_i,b_i)=\frac{1}{b_i^{a_i}\Gamma(a_i)}x_t^{a_i-1}e^{\frac{x_t}{b_i}} \quad \textbf{(2)}$$

$$\omega_{t,i}=\frac{\eta_i^0\ \gamma(x_t;a_i^0,b_i^0)}{\sum_j \eta_j^0\ \gamma(x_t;a_i^0,b_i^0)} \quad \textbf{(3)}$$

The probability of a gene being up-expressed given its expression level *x* is the probability of occurrence of the "up" state overall $p_{UP} = N_{UP}/N$ multiplied by the probability of expression level *x* corresponding to an up-expressed state or $\gamma(x;a_{UP}, b_{UP})$; where N_{UP} is the number of genes that are up-expressed among all N genes.

Every pathway consists of a collection of reaction steps or interactions. These can be modeled as logic functions with genes serving as inputs and outputs. In the current protocol the activation level of such a logical function was computed based on the joint conditional probability that the input genes $k \in$ set *I* are in an up-expressed state based on measured gene expression (Eq. 4). As we have estimated the corresponding probability that the set of output genes $k \in O$ are also in an up-expressed state (Eq. 5), we can use the agreement between input and output as a measure of consistency C_s for the activation of reaction step *s* (Eq. 6).

$$p(s=active)=\prod_{k\in I} p(g_k="up") \quad \textbf{(4)}$$

$$p(O="up")=\prod_{k\in O} p(g_k="up") \quad \textbf{(5)}$$

$$C_S = p(s = active) \times p(O = "up") + (1 - p(s = active)) \times (1 - p(O = "up")) \quad \textbf{(6)}$$

Using the approach outlined in Equations 1-6, activation levels and consistency were calculated in each patient sample for all reaction steps described in 582 pathways aggregated from the National Cancer Institute (NCI)-Nature Pathway Interaction Database (PID) (Schaefer et al., 2009) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2010). The NCI-Nature PID database is itself an aggregation of 135 pathways curated by the NCI-Nature team with an additional 322 pathways imported from the BioCarta (www.biocarta.com) and Reactome (Croft et al. 2011; Matthews et al. 2009) databases. In each individual sample the activity of each pathway was calculated as the average activation level across all component interactions. Activation scores in each subject group were log transformed to improve normality and compared for each pathway at each time point using both parametric (t test) and non-parametric (Wilcoxon rank sum) tests. Once again, two-way analysis of variance (ANOVA-2) was used to assess the significance of group, time and group x time interactions with the false discovery rate (FDR) estimated using Storey (2002). The potential of each pathway activation score as a diagnostic marker was described in terms of receiver-operating characteristics (ROC) by the increase above random in the area under the curve (AUC>0.50). All the computations were conducted with the MATLAB software environment (The MathWorks Inc., Natick, MA).

2.2.3 Identifying Patterns of Interaction

To describe in statistical terms the interactions linking the activity of individual pathways and measures of symptom severity we constructed empirical networks using

partial linear correlation as a measure of association (Emmert-Streib, 2007; Magwene and Kim, 2004). This measure adjusts the pair-wise correlation of pathway activity x_i with pathway activity x_j for the indirect correlation contributed by a third pathway x_k.

$$r(x_i, x_j \mid x_k) = \frac{r(x_i, x_j) - r(x_i, x_k)\, r(x_j, x_k)}{\sqrt{(1 - r^2(x_i, x_k))}\ \sqrt{(1 - r^2(x_j, x_k))}} \qquad \textbf{(7)}$$

In order to cast each pair-wise association in the context of the overall network we summarized the contribution of all remaining nodes by computing the first principal component for these nodes and using this as the context variable x_k (Equ. 7). Null probability p was computed by transforming the correlation to create a t statistic having n-2 degrees of freedom for n observations. Confidence bounds were based on an asymptotic normal distribution of 0.5*log((1+r(xixj |xk))/(1-r(xixj |xk))), with an approximate variance equal to 1/(n-3) when variables have a multivariate normal distribution. Once again the probability of false discovery q was estimated according to Storey (2002) with q<0.05 indicating significant associations. The graphical rendering of correlation networks as well as the analysis of network attributes was done using the Cytoscape platform (Smoot et al., 2011) with the NetworkAnalyzer component (Assenov et al., 2008).

3. Results.

3.1 Differential expression of transcription-associated markers.

The number of microarray probe sets changing in expression across time, subject group, or showing a group x time interaction with a probability of false discovery q<0.05 are shown in Table 2. Over 1,000 probes sets (1080) differed significantly in expression

across time in GWS. Using the PANTHER database this corresponded to 644 genes associated with 79 pathways. For the CFS/ME disease control group we found close to 300 probe sets (280) changing across time. These mapped to 180 genes that were in turn associated with 30 pathways. These changes across time were dwarfed by the very broad differences in expression observed across subject groups. Close to 9,000 probe sets (8819) were differentially expressed in GWS across all time points compared to the healthy control group. These mapped to 4404 genes associated with 126 pathways in the PANTHER database. In CFS/ME over 10,000 probe sets (10,222) changed significantly in expression compared with the healthy control group. These probe sets corresponded to 4,963 genes that in turn mapped onto 132 pathways. While significant effects were found for both time and group no probe sets presented significant time x group interactions with $q<0.05$.

3.2 Preferential activation of known pathways

Using the methodology proposed by Efroni et al. (2007, 2008) we obtained estimates of pathway activation levels for each individual sample by projecting gene expression onto the regulatory logic encoded by these pathways. As with the previous analysis conducted directly at the probe set level, a 2-way ANOVA of pathway activation found no significant time-group interactions. However unlike our probe-level analysis we also found that by enforcing adherence to a specific regulatory logic and aggregating across multiple pathway step reactions, changes across time were no longer discernable. As shown in Table 2, we found only group effects to be significant at the $q< 0.05$ level of confidence. In GWS we found 127 pathways to differ significantly in activation level compared to the healthy control group. This is comparable with the number found by relying on the gene and pathway annotation of the probe sets. The changes in 20 of

these pathways were found only in GWS and exceeded 10% of the corresponding levels found in the healthy control group (Table 3A). Results indicate decreased activation levels in several key processes regulating transcription, such as histone deacetylase (HDAC) signaling and kinase A-anchoring protein AKAP95 activity. We also observed changes in immune and neuro-immune activation. For example, we found reduced pathway activity for tumor necrosis factor receptor 1 (TNFr1) and FAS receptor signaling as well as tyrosine kinase mediated activation of T cell receptor signaling. This was accompanied by increased activation of pathways involving the broad-acting immune modulator NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Supplemental Figure S1). We also found significantly elevated activation of a KEGG super-pathway describing broad-scale receptor-ligand interactions for neurotransmitters such as acetylcholine, dopamine and others. This was accompanied by increased biosynthesis of phenylpropanoid compounds, a process promoted by the dopamine precursor phenylalanine. Finally, with possible ties to environmental exposure, we also observe chronic activation of the detoxification pathway supporting degradation of the androgen-disrupting hydrocarbon bisphenol.

For the disease control group CFS/ME we found 89 pathways that differed significantly ($q<0.05$) in activation level compared to the healthy control group; 25 of which changed in excess of 10% (Table 3B). These pathways show suppression of a variety of regulatory processes associated with transcription and cell cycle in CFS/ME. For example, estimated pathway activity supporting the role of protein kinase /cyclin A in centrosome duplication (Biocarta) is only one-tenth the level found in a typical healthy control subject. Indeed, activation level of the KEGG pathway for cell cycle constitutes a very strong potential classification marker in these subjects, producing a total area under the ROC curve in excess of 0.80 (or 0.32+0.50). Of note, several cancer-related

pathways including tumor suppression via p53 signaling, its interaction with estrogen-inducible RING finger protein (Efp) and hypoxia-inducible factor (pathway #192) as well as its stabilization by ATR (Ataxia-Telangiectasia/ Rad3 Related) in mediating breast cancer genes BRCA1/BRCA2 are all lowered in activation level. Conversely ephrin type-A2 (EPH A2) receptor signaling, involved in tumor development, migration and angiogenesis, appears overactive in this group (114% of healthy). Finally changes in energy metabolism were also observed in CFS/ME where a 50% increase in antioxidant lipoic acid metabolism (Manning et al., 2012) was accompanied by a 38% decrease in caffeine-induced lipolysis as well as leptin-mediated control of insulin resistance. At the cell-cell signaling level, these changes coincided with a significant decrease in pathway activation supporting B cell receptor signaling (86% of healthy).

While CFS/ME and GWS are commonly considered sister illnesses, we found little overlap at the level of pathway activation. Our analysis indicated that only 8 pathways were shared by both CFS/ME and GWS at a confidence level of $q<0.05$. Changes in these shared pathways were much more subdued with activity levels ranging from 93% to 110% of healthy control levels (see Supplemental Table S2). Control of skeletal myogenesis calmodulin-dependent kinase (CMAK) was the most over-expressed while the least expressed pathway supported granzyme A mediated apoptosis. ROC classification performance suggested that these shared pathways were generally better markers of CFS/ME than they were of GWS.

3.3 Pathway associations with symptom severity in GWS

The pathways described above are not expressed independently of one another but instead belong to a well-integrated biochemical network. To explore these coordinated interactions and their relevance to GWS we constructed networks linking individual

pathways and an ensemble of 27 indicators of symptom severity measured at rest as well as age, ethnicity and body-mass index (BMI) (Table S1). As some aspects of GWS may correlate best with exercise capacity we constructed separate association networks linking baseline symptom severity with pathway activation at rest, at peak effort and during the recovery phase (4 hours after peak effort). The structural characteristics of each network are listed in Supplemental Table S3. Results indicated that very few pathway or symptom nodes were orphaned with 444 to 460 of the 481 candidate nodes being connected to at least one neighbor at any point in time. Even using a partial correlation coefficient $|r|>0.10$ and a significance threshold of $q<0.01$ in pruning pathway-symptom associations, these networks were well-connected with a typical node counting up to 77 first neighbors. The networks did however fragment into components and consisted of up to 8 connected sub-assemblies at peak effort. As pathways far outnumbered symptoms, much of this structure consisted of pathway-pathway associations. With regard to the symptom nodes specifically, results in Figure 1(a) show that the bulk of the symptoms have fewer than 5 first neighbors, with most of these presenting at initial rest. A dramatic exception to this is the SF36 General score. With only one first neighbor at rest, this symptom node recruited 30 first neighbors at peak effort and retained 25 in the recovery phase. Connectivity to the broader network by association is captured formally by a measure called closeness centrality that scales between 0-1. Values reported in Figure 1(b) indicate that even though most symptoms do not have many first neighbors per se, they are invariably connected to hub nodes and are well integrated into the fabric of the pathway network especially when these are considered at peak effort or t(1).

Pathways that link directly to symptom nodes serve as an interface with the larger pathway network and deserve specific attention. Association networks showing only the

first neighbors of symptom nodes at initial rest, peak effort and recovery are presented graphically in Figure 2(a), (b) and (c). Only those symptom or demographic nodes having at least one pathway node as a first neighbor are shown. At initial rest t(0), we found 11 of the 24 symptom nodes supported a direct and significant association with a pathway node (see Supplemental Table S4). This dropped to 4 symptom nodes at peak effort and 6 symptom nodes for pathway activation measured in the recovery phase. While only age and BMI presented direct pathway associations at rest and peak effort respectively, all three demographic variables were associated with at least one pathway in the recovery phase. Age was the most prolific of these, being negatively correlated with a sub-network of 11 pathway nodes during recovery. One of these nodes, oxidant scavenging porphyrin and chlorophyll metabolism (Yu et al., 2010), was the only link via butonate metabolism to the motif consisting of MFI components for mental fatigue, general fatigue and reduced motivation. This same MFI triad correlates negatively with IL-10 anti-inflammatory signaling at initial rest.

Of the 8 components that constitute the SF36 Health Survey, three were associated directly to pathway activation at rest. Measures for general health and physical function were both tethered to a sub-network consisting of all 4 Davidson trauma measures and dominated by immune signaling pathways. SF36 general health correlated negatively with activation of naive CD8+ T cell signaling while SF36 physical function correlated positively with CXCR4 chemotatic signaling pathway and the complement/ coagulation cascade. SF36 social function score correlated positively with 4 pathways at rest, 3 of these were detoxification pathways for compounds commonly found in pesticides. Correlating negatively with IL-10 signaling and positively with scores for mental and general fatigue, the MFI score for reduced motivation was the only MFI component that associated directly with a pathway. The MFI score for physical fatigue was associated

with immune signaling processes only indirectly through a negative correlation with the Davidson intrusiveness score. Chondroitin sulfate synthesis showed a strong positive association with the Krupp fatigue severity score. Interestingly levels of chondroitin sulfate proteoglycans are vastly increased after injury to the central nervous system (Susarla et al., 2011). Scores for the Paced Auditory Serial Addition Task (PASAT) correlated negatively with activation of biotin or coenzyme R metabolism, a contributor to fatty acid synthesis and gluconeogenesis, and positively with ErbB protein or epidermal growth factor receptor (EGFR) signaling. Deficient ErbB signaling has been associated with neurodegenerative conditions such as Alzheimers (Bublil and Yarden, 2007).

Perhaps the most dramatic feature of symptom-pathway interaction in this data is the emergence of a large integrated sub-network of pathway nodes associated with SF36 general health score at peak effort and during recovery. This is the only symptom measure that correlates with changes in at least one pathway at all three time-points. This sub-network is comprised of 30 pathways at peak effort and 25 in recovery phase. Only 5 pathways are common to sub-networks at both time points: aspirin-induced inhibition of platelet activation, wnt signaling, lipoic acid metabolism, HDAC class I mediated signaling and streptomycin biosynthesis. All correlate negatively with SF36 general health score, more so at peak effort than during recovery (see Supplemental Table S5). The only other component of the SF36 inventory that correlated with pathway activation levels at peak effort t(1) and recovery t(2) was the score for emotional function. This component correlated negatively with the regulation of actin in cytoskeleton at t(1) and positively with BMI at t(2), through calcineurin-mediated keratinocyte differentiation and smad2/ smad3 transduction of TGF-β signaling. The same MFI motif consisting of general fatigue, reduced motivation and metal fatigue found at rest was also found at peak effort and recovery. These measures were

associated negatively with neuronal growth factor signaling via p75 neurotrophin receptor (NTR) at t(1) and positively with activation of butanoate metabolism and the age-centric metabolic pathway cluster at t(2).

Direct pathway associations with symptom clusters for both PASAT and Davidson trauma measures dissipated at peak effort only to reappear in the recovery phase. DTS measures clustered in a satellite sub-network in the recovery phase, tethered to the SF36 general health super-cluster via negative correlations to the regulation of autophagy, angiogenic-metabolic signaling involving phosphatidylinositol 3-kinase (PI3K) and AK Thymoma (Akt)/ Protein Kinase B (PKB) as well as via interactions between aspirin-mediated blocking of platelet activation and T cell receptor signaling. PASAT measures also clustered in a satellite sub-network attached to the same pathway super-cluster via negative correlation with biosynthesis of the antioxidant ubiquinone or coenzyme Q10. Direct associations of the Sickness Impact Profile (SIP) with pathway activation were only observed at peak effort. Interestingly SIP score presents strong negative correlations with 4 novel candidate pathways (pathways exp_1 through 4) sharing a similar structure. These describe interactions between PI3K, Akt/PKB and components of the EGF-ERK-ERF axis and were originally hypothesized as playing a role in angiogenesis and cell migration in breast cancer (Supplemental Figure S2) (Tarcic et al., 2011).

4. Discussion.

In this study we use a standard exercise challenge to stimulate immune and endocrine signaling in a group of veterans satisfying the case definition for Gulf War Syndrome (GWS) as well as a disease control group of non-veterans with chronic fatigue syndrome (CFS/ME) and healthy control subjects. Blood samples collected at initial rest,

at peak effort (VO2 max) and at 4 hours post effort were analyzed for gene expression in circulating lymphocytes using both a conventional single-gene approach and a more novel approach whereby transcript levels were projected onto the regulatory circuitry of known pathways. This projection resulted in a loss of resolution with respect to time effects however strong group effects were found with a number of pathways being uniquely expressed in GWS compared to both healthy controls and CFS/ME, an illness with similar clinical presentation. In GWS we found suppressed activity in roughly two thirds of the 20 affected pathways. Among these were processes involved in apoptosis signal transduction and elements of TNF receptor signaling. Conversely, activation of broad-scale ligand-receptor interactions supporting neurotransmission increased significantly in GWS as did that of master transcriptional regulator NF-κB. A key mediator of inflammatory response, the latter is up-regulated rapidly in healthy individuals during HPA-mediated response to stress by the release catecholamines, including the neurotransmitters dopamine and norepinephrine (Wolf et al., 2009). NF-κB is also involved in cholinergic signaling, controlling AChR clustering through transcriptional regulation of synaptic protein Rapsyn (Wang et al., 2010).

The suppression of apoptosis signal transduction in GWS contrasted sharply with CFS/ME where we found an almost opposite suppression of cell cycle progression, actin remodeling and metabolism including leptin's control of insulin resistance. Calcium-mediated activation of T cells, IFN signaling, B cell receptor signaling were also suppressed. This suppression of immune function may be further exacerbated by an over-activation of ephrin A (Eph-A) signaling in these subjects. Activation of Eph-A receptors in T cells is thought to inhibit SDF-1 (CXCL12)-induced chemotaxis by altering the balance of small GTPase activity (Arvanitis and Davy, 2008). Forward Eph-A signaling has also been linked to inhibition of insulin secretion (Pasquale, 2008)

underscoring the metabolic repercussions of abhorrent immune signaling. Finally, Eph-A interacts with TrkB to augment BDNF-promoted retinal axon branching (Marler et al., 2008). Interestingly Barbier et al. (2009) found alterations in gene expression for brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase B in stressed animals exposed to PB. Our results also suggest changes pathway activity supporting visual signal transduction in human GWS subjects (Table 3A) further emphasizing some of the commonalities linking these two illnesses.

Because individual pathways are part of an integrated cellular circuitry we constructed association networks based on partial correlation to map pathway-pathway interactions and their link to several measures of symptom severity in Gulf War Illness. This was not done for the CFS/ME group because of the small cohort size (n=7) leading to a narrow range of symptom severity. Of the 24 symptom and 3 demographic variables examined, 17 correlated directly in GWS with the activity of at least one pathway at one or another of the 3 exercise phases studied. Only one of these pathway interfaces was significantly altered in activity level in GWS: the NF-κB signaling pathway measured at rest. All other pathways differentially activated in GWS were embedded deeper in the interaction network and did not interface directly with symptom severity. Though their activation level was unremarkable in GWS, 4 symptom-pathway interface nodes identified at peak effort and recovery were significantly altered in expression in CFS/ME. Pathways for lipoic acid metabolism, calcium-mediated activation of NFAT (Nuclear factor of activated T-cells) transcription factor, cell cycle control at G1/S, aurora A signaling in mitosis and NF-κB activation (Linardopoulos, 2007) all correlated strongly with SF36 general health score in GWS. These were suppressed in activation level in CFS/ME with the exception of lipoic acid metabolism, which was significantly over-expressed.

Collectively, results of this analysis suggest that GWS and CFS/ME might be described at least in part by illness-specific alterations to selected components of the Wnt/ Ca2+/ NFAT/ NF-κB signaling axis and the intersecting PI3K, Akt/PKB, Eph (and mTOR) axis. Recent evidence from animal models combining stress and exposure to chemical agents has reinforced the potentially central role of cholinergic signaling in the pathology of GWS (Amourette et al., 2009; Barbier et al., 2009). Consistent with this, a variety of Wnt proteins have been shown to both inhibit (Wnt3a) (Wang et al., 2008) and stimulate (Wnt9a, 9b, 10b, 11 and 16) (Zhang et al., 2012) the clustering of acetylcholine receptors (AChRs). ACh signaling is now known to be a significant modulator of immune function (Kawashima and Fujii, 2003). Muscarinic acetylcholine signaling has been found to promote IL-8 release via PKC, ERK1/2 and NF-κB pathways (Profita et al., 2008). Likewise, signal transduction via nicotinic acetylcholine receptors (nAChRs) is known to activate NFAT in lymphocytes and endothelial cells, altering cellular growth and production of vascular endothelial growth factor (Oloris et al., 2010). Similarly, activation of nAChR by SLURP-1 leads to up-regulation of NF-κB expression via the Raf-1/MEK1/ERK1/2 cascade, which is mediated in part by Ca2+ dependent CaMKII /PKC activation (Chernyavsky et al., 2010). Recall that CaMKII was also up-regulated in stressed animals exposed to PB as was brain-derived neurotrophic factor (BDNF) (Barbier et al., 2009). In this analysis we found significant association (peak effort) of several MFI components with p75 signaling, a low-affinity receptor for BDNF. While nAChR response is initially attenuated (Fernandes et al., 2008), long-term exposure to BDNF up-regulates intracellular and surface pools of nAChR in the hippocampus (Massey et al., 2006). Finally, the reduced TNF-mediated signaling observed here can also be linked to the effects of chronic nAChR activation (Kawashima and Fujii, 2003).

In the networks presented here, symptom indicators were never more than 5 connecting pathway nodes away from the NF-κB pathway. Not surprisingly there is growing evidence linking altered cholinergic signaling and activity of this transcription factor with fatigue, cognitive decline and onset of symptoms found in post-traumatic stress disorder (PTSD). Loss of cholinergic input to the hippocampus has been shown to result in decreased PKA activity and increased NF-κB activity with loss of PKA-mediated glucocorticoid receptor expression (GR) (Lim et al., 2011). Correlating with Davidson trauma scores in this work, up-regulation of target genes for the NF-κB/Rel family of transcription factors has also been observed in monocytes of men with PTSD (O'Donovan et al., 2011) as well as in PBMC from women with childhood abuse-related PTSD (Pace et al., 2012). In animal models selective inhibition of this transcription factor significantly reduced the prevalence of extreme PTSD-like response to stress (Cohen et al., 2011). NF-κB signaling also plays a key role in mediating the actions of IL-1 and stress in the onset of depression-like behavior (Koo et al., 2010). Changes in the activity of IL-1R signal transduction, one of only 2 pathways representing IL-1 signaling in this dataset, were statistically significant (q=0.045) but the magnitude of these changes were less than 10% of normal. Similarly, in earlier work we found a strong influence of IL-1a on neuroendocrine-immune signaling in a subset of this GWS cohort even though levels in plasma did not differ significantly from control (Broderick et al., 2010).

Together with recent literature the data presented here supports an active role of cholinergic-immune signaling, either direct or indirect, in GWS. This correlates with observations of stress-potentiated exposure to ACh mediators (PB, organophosphates, sarin, tec…) in animal models indicating that these may have significant value as experimental systems for the continuing study of GWS. It is also important to note that many of the findings from this work would not have emerged from a conventional

analysis of gene expression levels alone emphasizing that the latter offers only a limited perspective of complex disorders like GWS.

Authors' contributions

Conceived and designed the experiments: MAF NGK GB. Performed the experiments: LN, MAF, NGK, ZB. Analyzed the data: RBH, GB, SV, SE. Contributed reagents/materials/analysis tools: SE, LN, MAF. Wrote the paper: GB, RBH, SE, NGK, MAF.

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Figure 1. *Symptom measures correlate significantly with pathway activation*. (A) Direct association of 24 symptom measures and 3 demographic variables (age, race and body-mass index (BMI)) with pathway activation nodes (first neighbors) in networks constructed on the basis of partial correlation coefficient. (B) Indirect association of demographic and symptom severity indicators with broader network via highly connected pathways nodes is increased at peak effort. Closeness centrality index is the reciprocal of the shortest path length to that node.

Figure 2. *Pathway sub-networks interface with symptom severity during exercise in GWI.* Association sub-networks in GWI showing only direct links between demographic and symptom nodes with pathway nodes at initial rest (A), at peak effort (B) and during recover, 4 hours post-effort (C). Links or edges indicate pair-wise partial correlations with a probability of false discovery $q \leq 0.01$; positive correlations are shown as green edges, negative correlations are shown as red edges.

Figure S1. *Inferred NF-κB activity during exercise*. Activity level of NF-κB signaling pathway (node 296) inferred from gene expression in circulating lymphocytes from GWS and CFS/ME subjects as well as healthy control subjects.

Figure S2. *Novel pathways exp_1-4*. Structure of novel pathways proposed by Tarcic et al. (2011) and hypothesized as playing a role in angiogenesis and cell migration in breast cancer.

Table(s)

Table 1. Summary of mean and standard error values ( ) for demographic variables and exercise performance in GWS, CFS/ME and healthy control groups.

		HC	CFS	GWS
Number of subjects		22	7	20
Race	Caucasian	4	6	7
	Hispanic	11	1	9
	African American	6	0	4
	Asian	1	0	0
Age (years)		41.2 (1.4)	45.7 (2.3)	42.7 (1.4)
Body-mass Index (BMI) (kg/m2)		27.9 (0.9)	26.2 (1.3)	28.3 (0.7)
Peak VO2 max (ml/kg/min)		27.6 (1.1)	21.7 (0.9) *	24.9 (1.0)
% Predicted Peak VO2 max (%)		76.0 (3.2)	63.7 (2.9) *	67.9 (2.4)

* 2-tailed t test significant at 0.05 level versus HC

Table(s)

Table 2. Summary of microarray probe sets with significant difference in expression level in GWS and CFS/ME groups compared to the healthy control group. Significance was based on p-values for time, group and time x group effects estimated from a 2-way ANOVA and set as p<0.05. Adjustment for multiple comparisons was based on Storey's q-statistic (q<0.05).

	p time <0.05	q <0.05	p group <0.05	q <0.05	p time x group <0.05	q < 0.05
Number of probe sets						
GWI vs HC all times	5313	1080	14510	8819	583	0
CFS vs HC all times	5008	280	15185	10222	3364	0
Number of genes (Unigene IDs mapped)						
GWI vs HC all times	2894	644	6682	4404	320	0
CFS vs HC all times	2951	180	6914	4963	1972	0
Number of pahways (from mapped IDs)	**Panther Database**					
GWI vs HC all times	114	79	138	126	65	0
CFS vs HC all times	112	30	137	132	109	0
Number of pathways (PID path activation)	**NCI/Nature PID**					
GWI vs HC all times	15	0	135	127	5	0
CFS vs HC all times	45	0	128	89	39	0

Table 3A. Summary of 20 pathways with significant difference in activity level unique to GWS compared to the CFS/ME and healthy control groups. Significance was based on $p<0.05$ for time, group and time x group effects estimated from a 2-way ANOVA and adjusted for multiple comparisons using Storey's q-statistic ($q<0.05$). Performance as potential diagnostic markers is shown as area under receiver-operator characteristic (ROC) curve (AUC) in excess of 0.50, the value expected from a random assignment.

Node #	Pathway #	Pathway Name	p group	q value	ROC AUC>0.50	*mean GWS/ mean Con*
37	10	akap95 role in mitosis and chromosome dynamics(biocarta)	0.000	0.006	0.173	0.75
460	433	tsp-1 induced apoptosis in microvascular endothelial cell(biocarta)	0.001	0.007	0.127	0.86
382	355	rna polymerase iii transcription(biocarta)	0.001	0.010	0.090	0.86
256	229	lck and fyn tyrosine kinases in initiation of tcr activation(biocarta)	0.003	0.021	0.072	0.86
404	377	signaling events mediated by hdac class iii(nci/nature)	0.009	0.030	0.083	0.88
263	236	lissencephaly gene (lis1) in neuronal migration and development(nci/nature)	0.043	0.038	0.101	0.89
159	132	fas signaling pathway (cd95)(biocarta)	0.004	0.016	0.102	0.89
101	74	caspase cascade in apoptosis(biocarta)	0.000	0.004	0.159	0.89
449	422	tnfr1 signaling pathway(biocarta)	0.003	0.011	0.083	0.89
160	133	fas signaling pathway (cd95)(nci/nature)	0.001	0.007	0.157	0.89
425	398	sumoylation by ranbp2 regulates transcriptional repression(nci/nature)	0.003	0.020	0.163	0.89
270	243	map kinase inactivation of smrt corepressor(biocarta)	0.024	0.042	0.114	0.90
394	367	rxr and rar heterodimerization with other nuclear receptor(nci/nature)	0.009	0.038	0.071	0.90
83	56	bisphenol a degradation(kegg)	0.007	0.022	0.128	1.11
292	265	neuroactive ligand-receptor interaction(kegg)	0.004	0.014	0.133	1.12
421	394	stress induction of hsp regulation(biocarta)	0.000	0.006	0.195	1.13
332	305	phenylpropanoid biosynthesis(kegg)	0.016	0.032	0.136	1.18
65	38	atypical nf-kappab pathway(nci/nature)	0.001	0.006	0.172	1.21
476	449	visual signal transduction: cones(nci/nature)	0.032	0.044	0.111	1.26
296	269	nf-kb signaling pathway(biocarta)	0.000	0.006	0.170	1.33

Table 3B. Summary of 25 pathways with significant difference in activity level unique to CFS/ME compared to the GWS and healthy control groups.

Node #	Pathway #	Pathway Name	p group	q value	ROC AUC>0.50	*mean CFS/ mean Con*
348	321	protein kinase a at the centrosome(biocarta)	0.000	0.002	0.256	0.11
156	129	estrogen responsive protein efp controls cell cycle and breast tumors growth(biocarta)	0.000	0.002	0.185	0.42
316	289	p53 signaling pathway(kegg)	0.000	0.002	0.216	0.62
315	288	p38 signaling mediated by mapkap kinases(nci/nature)	0.000	0.001	0.024	0.67
379	352	reversal of insulin resistance by leptin(biocarta)	0.007	0.023	0.172	0.72
92	65	caffeine metabolism(kegg)	0.003	0.010	0.165	0.72
383	356	role of brca1 brca2 and ATR in cancer susceptibility(biocarta)	0.000	0.000	0.357	0.73
374	347	regulation of transcriptional activity by pml(biocarta)	0.000	0.002	0.212	0.75
364	337	regulation of cell cycle progression by plk3(biocarta)	0.000	0.000	0.283	0.77
93	66	calcineurin-regulated nfat-dependent transcription in lymphocytes(nci/nature)	0.002	0.008	0.199	0.79
106	79	cell cycle: g1/s check point(biocarta)	0.000	0.002	0.207	0.80
384	357	role of calcineurin-dependent nfat signaling in lymphocytes(nci/nature)	0.000	0.001	0.315	0.82
59	32	arf6 signaling events(nci/nature)	0.000	0.002	0.022	0.83
475	448	visual signal transduction(biocarta)	0.003	0.010	0.200	0.84
437	410	tetrachloroethene degradation(kegg)	0.005	0.016	0.222	0.85
249	222	integrins in angiogenesis(nci/nature)	0.000	0.001	0.280	0.86
70	43	b cell receptor signaling pathway(kegg)	0.004	0.014	0.194	0.86
111	84	chaperones modulate interferon signaling pathway(biocarta)	0.000	0.002	0.185	0.86
66	39	aurora a signaling(nci/nature)	0.001	0.003	0.231	0.87
354	327	rac1 cell motility signaling pathway(biocarta)	0.000	0.000	0.315	0.87
400	373	signaling by aurora kinases(nci/nature)	0.002	0.008	0.105	0.88
105	78	cell cycle(kegg)	0.000	0.000	0.320	0.90
219	192	hypoxia and p53 in the cardiovascular system(biocarta)	0.000	0.002	0.185	0.90
144	117	epha2 forward signaling(nci/nature)	0.018	0.044	0.182	1.14
261	234	lipoic acid metabolism(kegg)	0.011	0.032	0.126	1.52

Table S1. Summary of mean and standard error values ( ) for measures of symptom severity in GWS, CFS/ME and healthy control groups. The significance of pair-wise comparisons is shown as p-values corresponding to a two-tailed t test (or Z test for DTS).

		HC (n=22)	CFS (n=7)	GWS (n=20)	*p* CFS vs HC	*p* GWS vs HC	*p* CFS vs GWS
Short Form SF36 Survey	Vitality	72.5 (2.4)	61.4 (4.6)	62.0 (3.1)	0.061	0.011	0.919
	Physical Function	83.8 (6.1)	42.1 (8.7)	58.4 (7.3)	0.002	0.013	0.176
	Physical Limit	78.6 (8.7)	- (-)	27.5 (7.5)	0.000	-	-
	Emotional Limit	93.3 (5.2)	71.4 (15.3)	28.3 (8.5)	0.215	0.000	0.034
	Emotional wellness	58.4 (1.0)	48.6 (2.7)	52.0 (1.5)	0.009	0.002	0.293
	Social Function	87.5 (5.5)	28.6 (9.3)	48.7 (6.9)	0.000	0.000	0.109
	Pain	76.2 (6.9)	32.9 (11.7)	40.8 (5.1)	0.009	0.000	0.552
	General	66.7 (2.8)	38.6 (4.2)	41.8 (2.7)	0.000	0.000	0.535
Multidimensional Fatigue Inventory (MFI)							
	General Fatigue	20.8 (4.4)	76.8 (8.3)	65.4 (5.3)	0.000	0.000	0.270
	Physical Fatigue	20.8 (4.9)	73.8 (9.2)	55.7 (6.3)	0.001	0.000	0.135
	Mental Fatigue	13.7 (4.3)	70.2 (4.9)	62.2 (6.1)	0.000	0.000	0.318
	Reduced Activity	19.9 (4.0)	60.8 (10.5)	51.5 (6.4)	0.007	0.001	0.470
	Reduced Motivation	16.7 (4.5)	40.4 (9.2)	54.9 (6.7)			
Krupp Fatigue Severity Inventory (Krupps)		18.1 (2.3)	57.3 (1.3)	44.9 (3.2)	0.000	0.000	0.002
Sickness Impact Profile (SIP)		7.2 (2.9)	31.1 (5.8)	36.9 (5.9)	0.005	0.000	0.499
Pittsburgh Sleep Quality Index (PSQI)		5.1 (1.0)	13.1 (0.8)	11.8 (0.9)	0.000	0.000	0.308
Davidson Trauma Scale (DTS) [Z test **]							
	DTS Total	*n=4 in 22> 0*	*n= 1in 7 >0*	78.3 (7.3)	-	0.000	-
	Intrusiveness Score	-	-	22.3 (2.3)	-	0.000	-
	Avoidance/Numbness	-	-	31.3 (3.5)	-	0.000	-
	Hyperarous	-	-	24.6 (2.0)	-	0.000	-
Paced Auditory Serial Addition Task (PASAT)							
	Trial 1	39.8 (3.0)	43.0 (3.4)	31.4 (3.1)	0.275	0.066	0.033
	Trial 2	37.6 (3.1)	39.5 (3.7)	28.9 (2.5)	0.693	0.037	0.041
	Trial 3	32.3 (2.4)	37.5 (2.8)	24.3 (2.7)	0.177	0.033	0.013
	Trial 4	23.5 (1.7)	25.8 (1.9)	18.9 (2.3)	0.366	0.122	0.034

Table S2. Summary of 8 pathways with significant difference in activity level common to GWS and CFS/ME compared to the healthy control group. Significance was based on p-values for time, group and time x group effects estimated from a 2-way ANOVA and set as $p<0.05$. Adjustment for multiple comparisons was based on Storey's q-statistic ($q<0.05$). Performance as potential diagnostic markers is shown as area under receiver-operator characteristic (ROC) curve (AUC) in excess of 0.50, the value expected from a random assignment.

			CFS			***GWS***				
Node #	**Pathway #**	**Pathway Name**	**p group**	**q value**	**ROC AUC>0.50**	**p group**	**q value**	**ROC AUC>0.50**	***mean CFS/ mean Con***	***mean GWS/ mean Con***
121	94	control of skeletal myogenesis by hdac and calcium/calmodulin-dependent kinase (camk)(biocarta)	0.020	0.049	0.170	0.008	0.043	0.153	1.10	1.09
96	69	canonical nf-kappab pathway(nci/nature)	0.007	0.022	0.157	0.003	0.020	0.177	1.06	1.05
403	376	signaling events mediated by hdac class ii(nci/nature)	0.018	0.044	0.147	0.000	0.011	0.208	1.04	1.05
377	350	repression of pain by transcriptional regulator dream(biocarta)	0.008	0.025	0.247	0.002	0.020	0.096	0.98	0.95
353	326	pyruvate metabolism(kegg)	0.000	0.000	0.302	0.022	0.050	0.092	0.95	0.98
473	446	vegfr3 signaling in lymphatic endothelium(nci/nature)	0.016	0.041	0.170	0.012	0.036	0.095	0.94	0.96
410	383	signaling mediated by p38-alpha and p38-beta(nci/nature)	0.014	0.036	0.185	0.008	0.030	0.169	0.94	0.93
205	178	granzyme a mediated apoptosis pathway(biocarta)	0.006	0.020	0.199	0.007	0.021	0.163	0.94	0.95

Table(s)

Table S3. Summary of network properties* for sub-networks consisting of symptom indicators, demographic variables and directly associated pathway nodes.

Network attributes (q<0.01)	Initial rest t(0)	Peak effort t(1)	Recovery phase t(2)
Size			
No. of Connected Nodes	458	444	460
No. of Edges (q<0.01, abs(r)>0.10)	14917	10974	17795
Local connectivity			
Avg. No. of Neighbours	65.14	49.43	77.21
Network density	0.14	0.11	0.17
Clustering Coeff.of neighbors	0.57	0.58	0.61
Granularity			
Connected Sub-assemblies	3	8	6
Network centralization	0.29	0.26	0.30
Network heterogeneity	0.87	0.93	0.84
Breadth			
Characteristic path length	2.51	2.66	2.34
Network diameter	8	9	7

Network attributes (q<0.05)	Initial rest t(0)	Peak effort t(1)	Recovery phase t(2)
Size			
No. of Connected Nodes	472	469	476
No. of Edges (q<0.05, abs(r)>0.10)	29877	24545	32314
Local connectivity			
Avg. No. of Neighbours	126.60	104.67	135.77
Network density	0.27	0.22	0.29
Clustering Coeff.of neighbors	0.61	0.60	0.64
Granularity			
Connected Sub-assemblies	1	2	1
Network centralization	0.31	0.30	0.28
Network heterogeneity	0.65	0.72	0.65
Breadth			
Characteristic path length	1.86	2.02	1.85
Network diameter	5	5	5

* *Network density*: the average number of first neighbors adjusted for network size. *Clustering coefficient*: the number of connected pairs in this subset of neighbor nodes. Number of *Connected sub-assemblies* indicates overall granularity. *Network centralization* and *Network heterogeneity*: describe the tendency of a network to contain hub nodes. *Network centralization*: similarity to a star configuration (centralization=1). *Characteristic path length and network diameter*: Respectively the average shortest and longest path length separating any two nodes.

Table S4. Summary of the number of symptom and demographic variable first neighbor nodes (Total Neigh.) and first neighbor *pathway* nodes (1st Neigh.).

Label	Node Name	Initial rest t(0)		Peak effort t(1)		Recovery t(2)	
		Total Neigh.	*1st Neigh.*	*Total Neigh.*	*1st Neigh.*	*Total Neigh.*	*1st Neigh.*
Age		1	1			11	11
Race						2	2
BMI				1	1	1	1
	Short Form 36 Health Survey (SF36)						
SF36_V	SF36_Vitality	1		1			
SF36_PF	SF36_Phys Func	1	1				
SF36_PL	SF36_Phys.Limit						
SF36_EL	SF36_Emot Limit			1	1	1	1
SF36_EW	SF36_Emot well						
SF36_SF	SF36_Social Fx	4	4				
SF36_P	SF36_Pain	1		1			
SF36_G	SF36_General	1	1	30	30	25	25
	Multidimensional Fatigue Inventory (MFI)						
MFI_GF	MFI_Gen Fatig	1		1		1	
MFI_PF	MFI_Phys Fatig	2		1		2	
MFI_MF	MFI_Ment Fatig	1		2	1	1	
MFI_RA	MFI_Red Act						
MFI_RM	MFI_Red Mot	3	1	2		3	1
Krupps	Krupp Fatigue Severity Inventory (Krupps)	1	1				
SIP	Sickness Impact Profile (SIP)	1		4	4		
PSQI	Pittsburgh Sleep Quality Index (PSQI)						
	Davidson Trauma Scale (DTS)						
DTS_T	DTS Total	6	3	3		3	
DTS_IS	DTS_Intrusiveness Score	12	8	2		7	3
DTS_AN	DTS_Avoidance/Numbness	3	1	2		2	
DTS_H	DTS_Hyperarous	3		2		3	
PASAT1	Paced Auditory Serial Addition Task 1(PASAT)	3		3		3	
PASAT2	Paced Auditory Serial Addition Task 2(PASAT)	3	1	2		4	2
PASAT3	Paced Auditory Serial Addition Task 3(PASAT)	4	1	3		5	2
PASAT4	Paced Auditory Serial Addition Task 4(PASAT)	4	2	2		2	

Table S5. Pathway nodes acting as first neighbors to SF36 General Health score at initial rest t(0), peak effort t(1) and 4 hours post effort t(2).

Network Node #	PID Pathway #	Node Name	Partial Corr. Coeff. *r*
Initial rest t(0)			
134	107	downstream signaling in naïve cd8+ t cells(nci/nature)	-0.661
Peak effort t(1)			
34	7	adherens junction(kegg)	0.789
202	175	glypican pathway(nci/nature)	0.770
433	406	taurine and hypotaurine metabolism(kegg)	0.739
464	437	tyrosine metabolism(kegg)	0.724
41	14	alkaloid biosynthesis i(kegg)	0.724
135	108	drug metabolism - cytochrome p450(kegg)	0.718
120	93	complement and coagulation cascades(kegg)	0.716
61	34	arginine and proline metabolism(kegg)	0.715
201	174	glypican 3 network(nci/nature)	0.708
331	304	phenylalanine, tyrosine and tryptophan biosynthesis(kegg)	0.704
375	348	regulators of bone mineralization(biocarta)	0.692
36	9	agrin in postsynaptic differentiation(biocarta)	0.685
477	450	visual signal transduction: rods(nci/nature)	0.681
129	102	d-arginine and d-ornithine metabolism(kegg)	0.680
93	66	calcineurin-regulated nfat-dependent transcription in lymphocytes(nci/nature)	0.676
87	60	btg family proteins and cell cycle regulation(biocarta)	0.673
384	357	role of calcineurin-dependent nfat signaling in lymphocytes(nci/nature)	0.671
104	77	cell adhesion molecules (cams)(kegg)	0.669
199	172	glycosylphosphatidylinositol(gpi)-anchor biosynthesis(kegg)	-0.679
241	214	inhibition of cellular proliferation by gleevec(biocarta)	-0.706
299	272	nitrogen metabolism(kegg)	-0.711
346	319	propanoate metabolism(kegg)	-0.713
165	138	fc epsilon ri signaling pathway(kegg)	-0.722
356	329	ras signaling pathway(biocarta)	-0.726
367	340	regulation of eif2(biocarta)	-0.743
63	36	aspirin blocks signaling pathway involved in platelet activation(biocarta)	-0.745
97	70	canonical wnt signaling pathway(nci/nature)	-0.675
261	234	lipoic acid metabolism(kegg)	-0.686
402	375	signaling events mediated by hdac class i(nci/nature)	-0.686
420	393	streptomycin biosynthesis(kegg)	-0.777

Table S5 (cont'd). Pathway nodes acting as first neighbors to SF36 General Health score at initial rest t(0), peak effort t(1) and 4 hours post effort t(2).

Network Node #	PID Pathway #	Node Name	Partial Corr. Coeff. *r*
Recovery phase t(2)			
172	145	fosb gene expression and drug abuse(biocarta)	0.749
459	432	tryptophan metabolism(kegg)	0.701
124	97	cxcr4 signaling pathway(biocarta)	0.671
212	185	histidine metabolism(kegg)	0.664
358	331	rb tumor suppressor/checkpoint signaling in response to dna damage(biocarta)	0.661
106	79	cell cycle: g1/s check point(biocarta)	0.651
66	39	aurora a signaling(nci/nature)	0.645
350	323	pten dependent cell cycle arrest and apoptosis(biocarta)	0.644
362	335	regulation of autophagy(kegg)	-0.644
436	409	terpenoid biosynthesis(kegg)	-0.653
73	46	basal cell carcinoma(kegg)	-0.654
116	89	class i pi3k signaling events mediated by akt(nci/nature)	-0.654
166	139	fc-epsilon receptor i signaling in mast cells(nci/nature)	-0.654
164	137	fc epsilon receptor i signaling in mast cells(biocarta)	-0.656
469	442	valine, leucine and isoleucine degradation(kegg)	-0.660
215	188	hiv-1 nef: negative effector of fas and tnf-alpha(nci/nature)	-0.661
211	184	hif-1-alpha transcription factor network(nci/nature)	-0.665
38	11	akt signaling pathway(biocarta)	-0.672
376	349	renal cell carcinoma(kegg)	-0.684
245	218	insulin signaling pathway(biocarta)	-0.752
63	36	aspirin blocks signaling pathway involved in platelet activation(biocarta)	-0.653
97	70	canonical wnt signaling pathway(nci/nature)	-0.667
261	234	lipoic acid metabolism(kegg)	-0.659
402	375	signaling events mediated by hdac class i(nci/nature)	-0.671
420	393	streptomycin biosynthesis(kegg)	-0.701

Figure(s)

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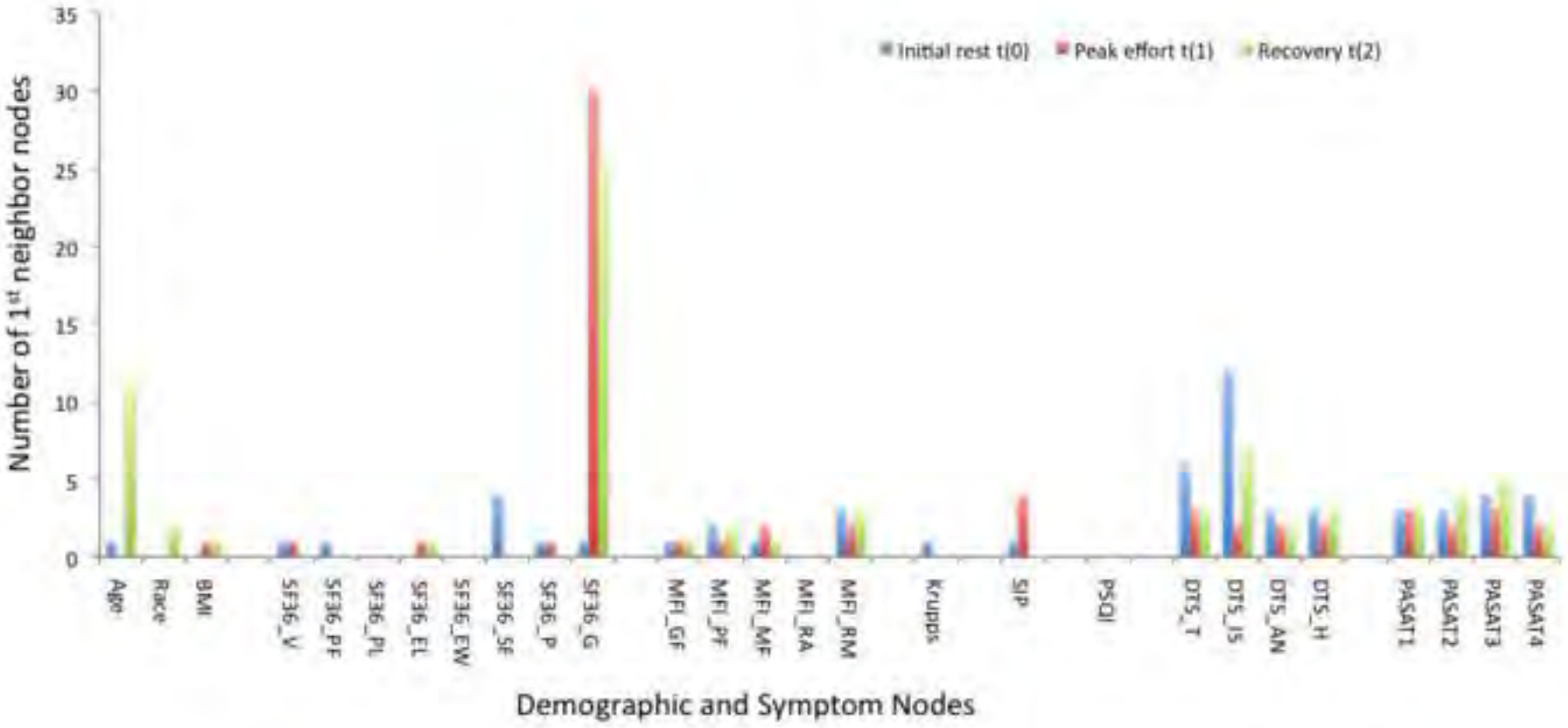

Fig 1A.

Figure(s)

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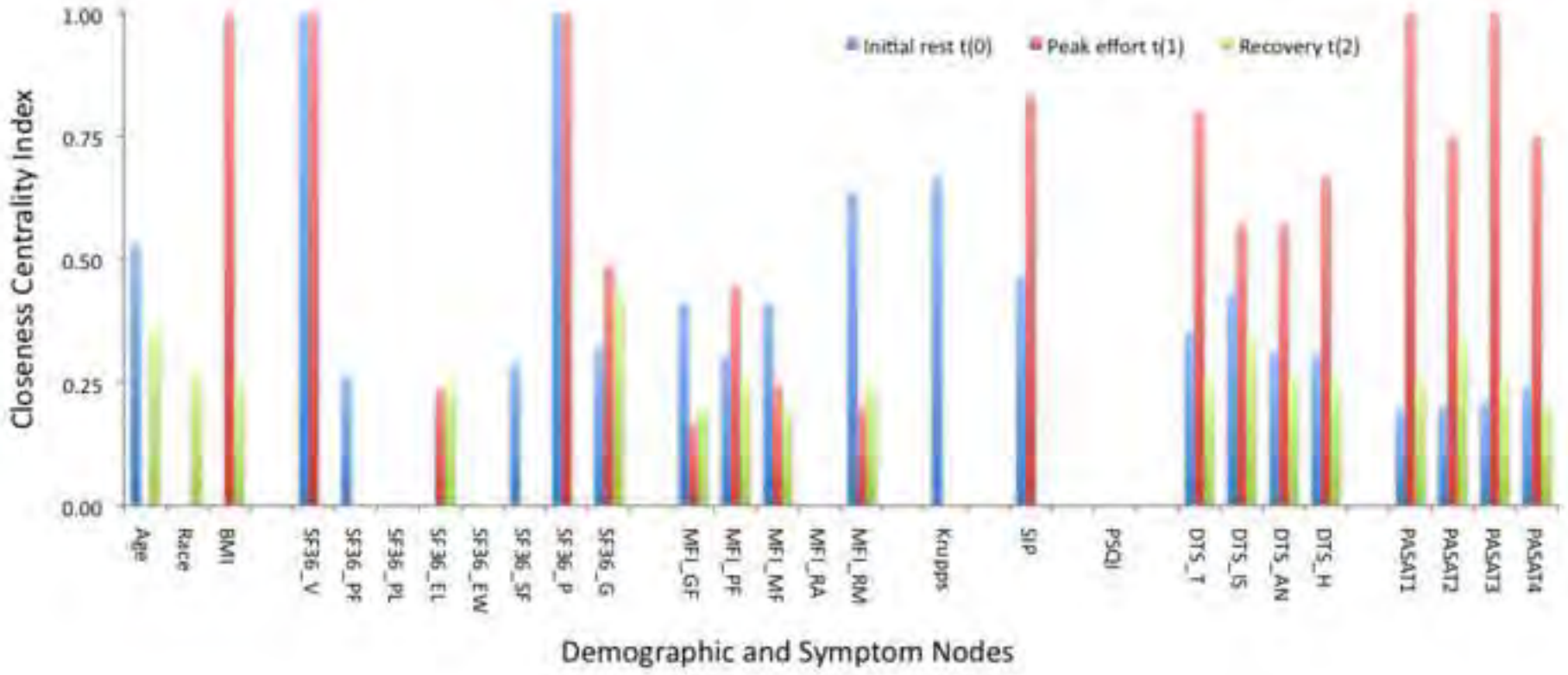

Fig 1B.

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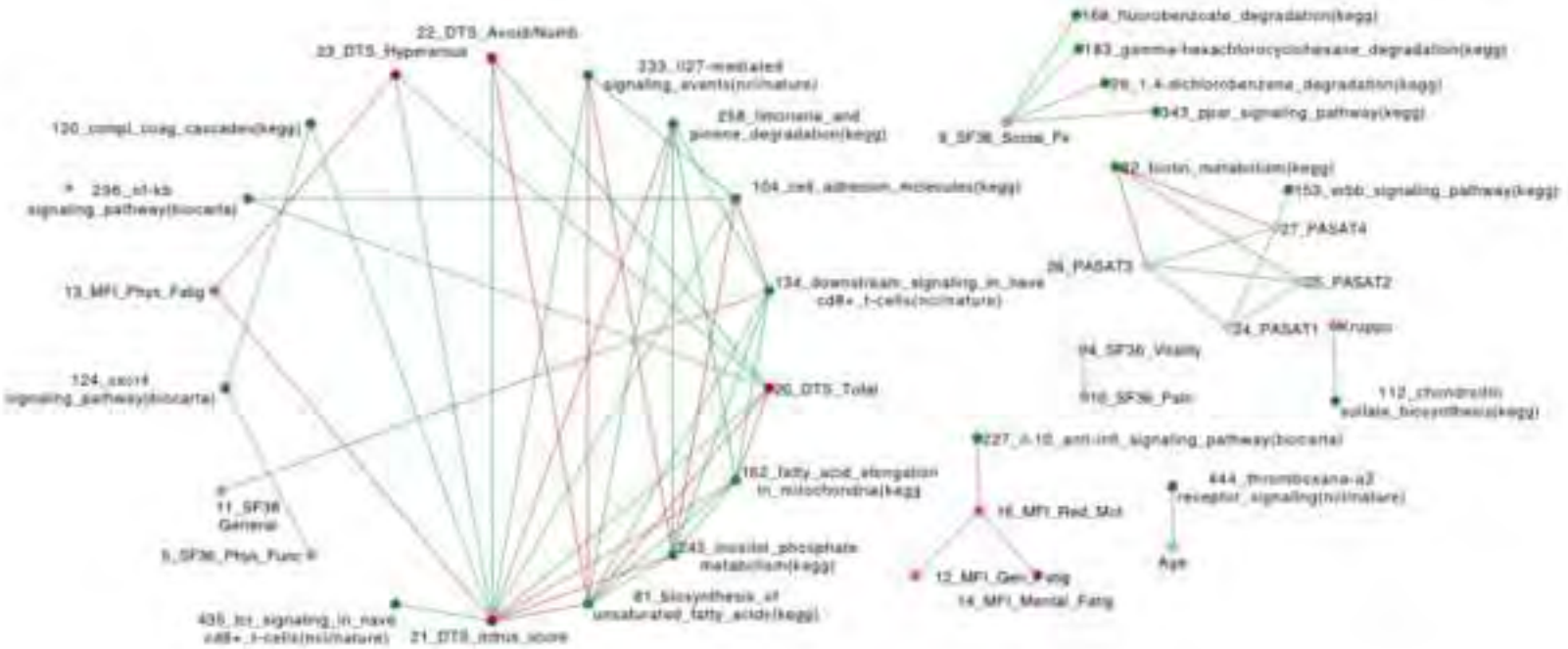

* Expressed in GWS vs Control at q<0.05

Fig 2A.

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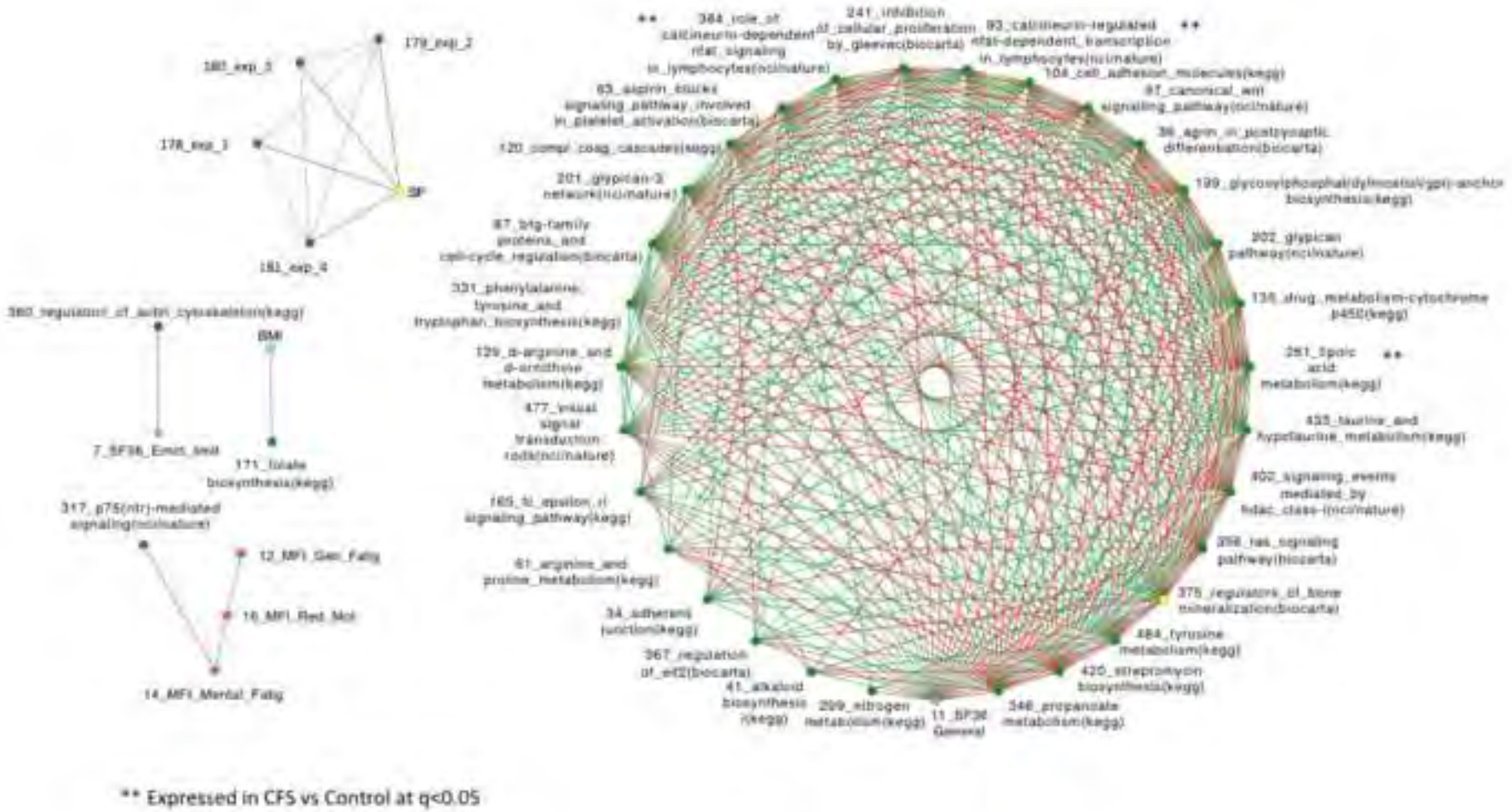

** Expressed in CFS vs Control at q<0.05

Fig 2B.

Figure(s)

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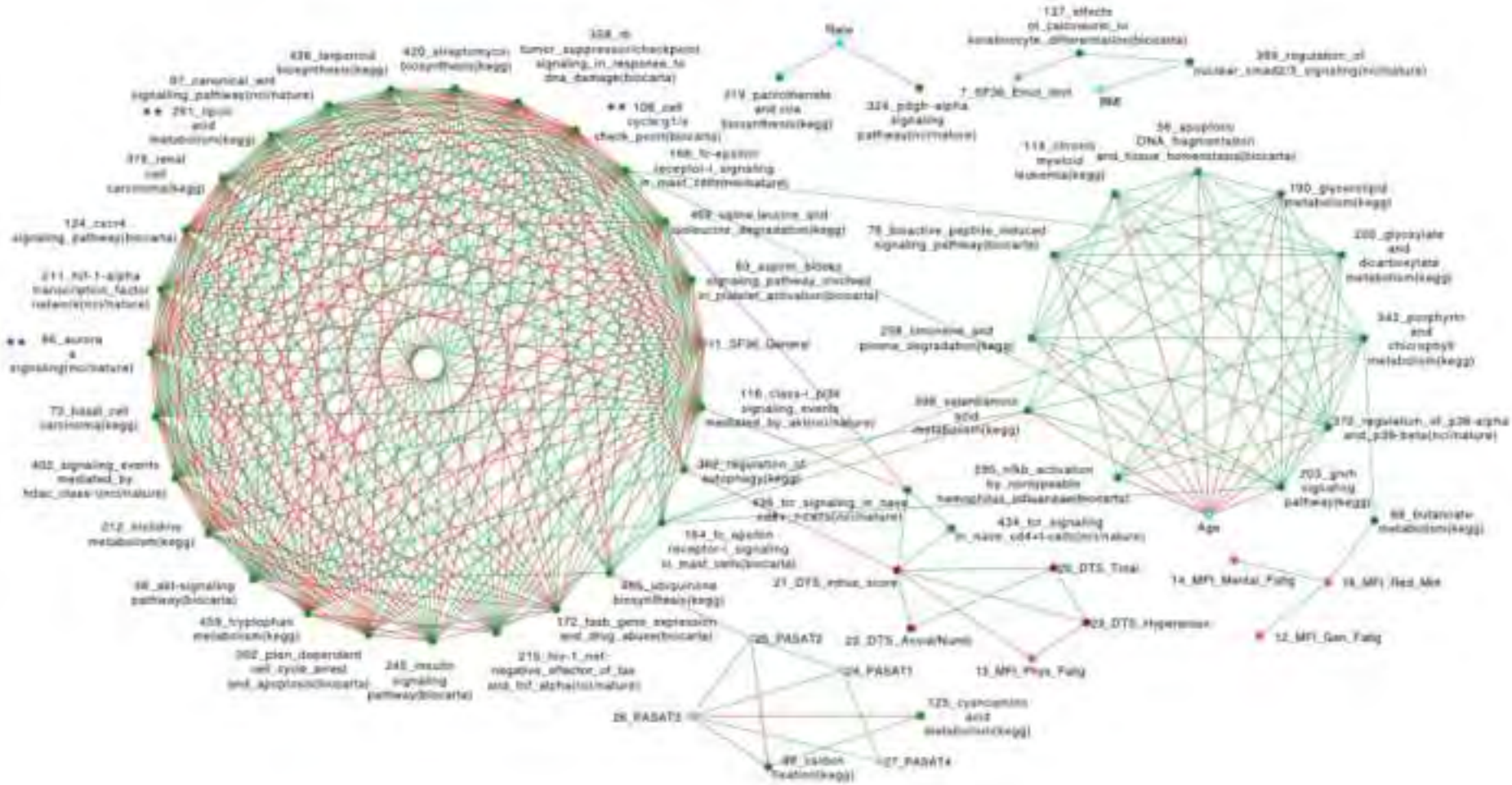

Fig 2C.

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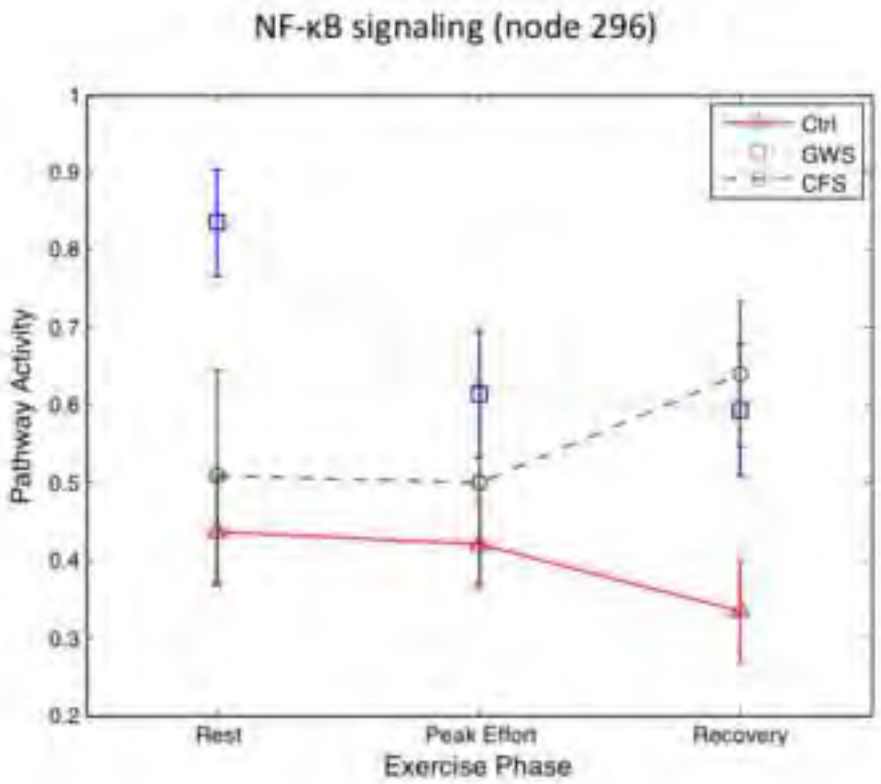

Fig S1.

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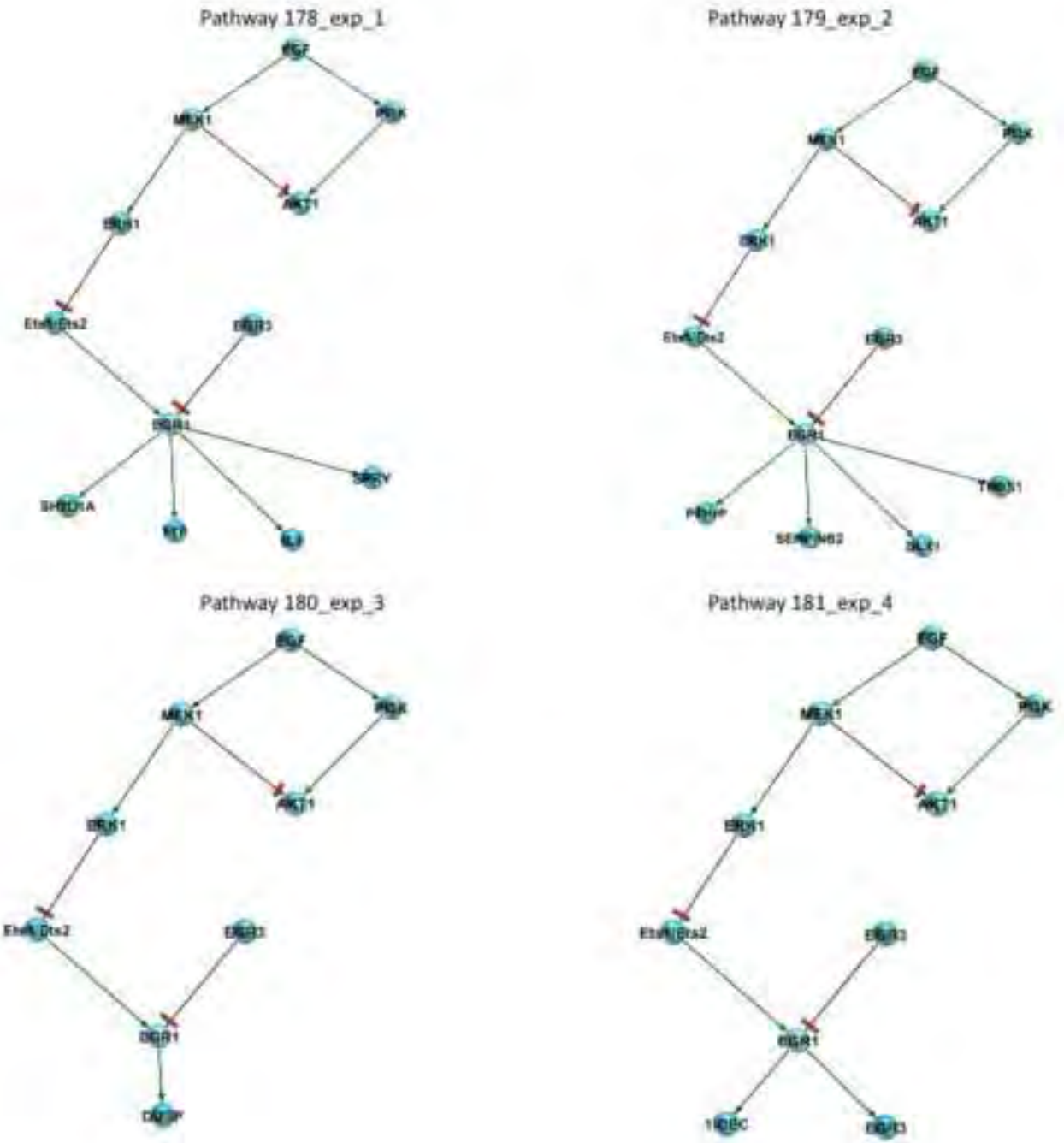

Fig S2.